*10/584960*

## Description

# MODULATION OF EPIDERMAL GROWTH FACTOR
# HETERODIMER ACTIVITY

5                          Cross Reference to Related Applications

This application claims the benefit of U.S. Provisional Patent Application Serial No. 60/545,185, filed February 17, 2004, the contents of which is herein incorporated by reference in its entirety.

10                                   Technical Field

The presently disclosed subject matter relates generally to the modulation of the activity of a heterodimer of an epidermal growth factor receptor (EGFR) with other members of the ERBB family in the regulation of cellular proliferation, differentiation, and survival. More particularly, the

15      presently disclosed subject matter relates to the modulation of the activity of EGFR/ERBB2, EGFR/ERBB3, and/or EGFR/ERBB4 heterodimers in the regulation of cellular proliferation, differentiation, and survival.

                                     Grant Statement

                                     Background Art

25          The epidermal growth factor receptor (EGFR) is the prototypical type-I receptor tyrosine kinase and the first member of the ERBB family of receptors to be identified (Coussens et al., 1985; Kraus et al., 1989; Carpenter & Wahl, 1990; Plowman et al., 1990; Plowman et al., 1993; Gullick, 1998). It is a member of the ERBB family of receptor tyrosine

30      kinases, and is also known as HER1 and ERBB1. Other members of the family include HER2/NEU/ERBB2, HER3/ERBB3, and HER4/ERBB4.

The ERBB receptors have similar structures, with extracellular ligand-binding domains, a single membrane-spanning region, and a cytoplasmic

protein tyrosine kinase domain. Under normal physiological conditions, the activity of the ERBB receptors is controlled by the availability of their ligands, which are members of the EGF-related growth factor family. The binding of EGF to EGFR causes dimerization of the receptor, leading to autophosphorylation and the recruitment of various molecules associated with signal transduction. Ligand binding to EGFR also results in the formation of heterodimers of EGFR and other members of the ERBB family.

EGFR, like other ERBB receptors, is required for normal mammalian development (Threadgill et al., 1995). However, inappropriate expression and/or activity of the ERBB receptors has been associated with various tumors, including tumors of the breast, colon, lung, ovary, and head and neck, and also in glioma (Rasheed et al., 1999; Wong et al., 1992; Moscatello et al., 1995). For example, there is some indication that the activity of heterodimers between EGFR and other ERBB family members (for example, ERBB2) is associated with cellular transformation and poor prognosis. Additionally, ERBB2 must heterodimerize with another ERBB family member in order to become activated, and given the role that ERBB2 appears to play in metastatic breast cancer, an ability to modulate the formation of ERBB2-containing heterodimers would be a possible strategy for treating and/or preventing this disease.

As such, there is considerable effort in the medical community to identify modulators of the ERBB family. Several such modulators are in clinical development or are already available, including the anti-EGFR antibodies ERBITUX® (cetuximab; ImClone Systems Incorporated, New York, New York, United States of America) and ABX-EGF (panitumumab; Abgenix, Inc., Fremont, California, United States of America). HERCEPTIN® (trastuzumab; Genentech, Inc., South San Francisco, California, United States of America) is a monoclonal antibody directed against ERBB2/HER2 that has been approved by the Food and Drug Administration for the treatment of ERBB2/HER2 positive metastatic breast cancer. Each of these antibodies is believed to modulate receptor activity by blocking ligand binding and/or enhancing endocytosis of the receptor, thus limiting its availability to become activated by ligand binding.

Small molecule inhibitors of EGFR, including IRESSA® (gefitinib/ZD1839; AstraZeneca PLC, London, United Kingdom) and TARCEVA™ (erlotinib; (OSI)™ Pharmaceuticals, Melville, New York, United States of America), have also been produced. These small molecules are thought to inhibit the kinase activity of EGFR. However, they are likely to be non-specific for EGFR, with significant activity on other tyrosine kinases.

Despite these advances, current methods for modulating EGFR heterodimer activity are hindered by their reliance on modulators that also modulate the activity of homodimers of the ERBB family and/or other tyrosine kinases. Ideally, a modulator should modulate the activity of EGFR heterodimers without affecting the activity of homodimeric forms of the ERBB family or ERBB family heterodimers that do not include EGFR. Thus, there exists a long-felt need in the art for modulators that are specific for heterodimers that contain EGFR.

To meet this need, the presently disclosed subject matter provides in some embodiments a method for identifying modulators that specifically bind to EGFR heterodimers. Such modulators are useful for treating disorders associated with undesirable EGFR heterodimer activity, among other applications.

## Summary

The presently disclosed subject matter provides a method for screening a plurality of compounds for an ability to bind to a heterodimer of EGFR and another ERBB family member. In some embodiments, the method comprises (a) contacting a first structure comprising an EGFR/ERBB heterodimer with a first solution, the first solution comprising the plurality of compounds; (b) removing any compounds bound to the first structure to produce a second solution; (c) contacting a second structure comprising an EGFR homodimer with the second solution, wherein the first structure and the second structure are identical except that the second structure does not contain an ERBB family member other than EGFR; and (d) recovering any unbound compounds to produce a third solution, whereby a compound that binds to a heterodimer of EGFR and another ERBB family member is

identified. In some embodiments, the plurality of compounds comprises a plurality of antibodies. In some embodiments, the plurality of compounds comprises phage-displayed antibodies. In some embodiments, the plurality of compounds comprises a phage-displayed antibody library. In some embodiments, the phage-displayed antibody library comprises a phage-displayed single chain variable fragment (scFv) library or a phage-displayed Fab library. In some embodiments, the phage-displayed antibodies are humanized.

In some embodiments of the present method, the first structure and the third structure comprise a cell that expresses EGRF and another ERBB family member, or an isolated membrane fraction of said cell. In some embodiments, the cell is a recombinant cell that does not normally express any ERBB family member or ErbB ligand, but has been engineered to express a human EGFR and at least one other human ERBB family member. In still another embodiments, the second structure comprises a cell that expresses EGRF but no other ERBB family member, or an isolated membrane fraction of said cell. In some embodiments, the cell is a recombinant cell that does not normally express any ERBB family member or ErbB ligand, but has been engineered to express a human EGFR.

In some embodiments, the method further comprises contacting a third structure comprising an EGFR/ERBB heterodimer with the third solution; and detecting binding of a compound to the EGFR/ERBB heterodimer on the third structure. In some embodiments, the method further comprises negatively selecting the plurality of compounds by contacting the plurality of compounds with a structure that is identical to the first and second structures except that it does not contain any ERBB family members.

The presently disclosed subject matter also provides a compound identified by the disclosed methods. Such compounds can be employed for treating any disorder associated with undesirable EGFR heterodimer activity, for example, a tumor associated with EGFR heterodimer activity. Thus, the presently disclosed subject matter also provides a method for suppressing the growth of a tumor associated with EGFR heterodimer activity in a

subject. In some embodiments, the method comprises administering to the subject bearing the tumor associated with EGFR heterodimer activity an effective amount of a compound identified by the disclosed methods, whereby growth of the tumor is suppressed. In some embodiments, the

5    compound comprises an antibody or antibody fragment. In some embodiments, the antibody or antibody fragment is a single chain fragment variable (scFv) antibody or an Fab antibody. In some embodiments, the tumor is selected from the group consisting of benign intracranial meningiomas, arteriovenous malformation, angioma, macular degeneration,

10   melanoma, adenocarcinoma, malignant glioma, prostatic carcinoma, kidney carcinoma, bladder carcinoma, pancreatic carcinoma, thyroid carcinoma, lung carcinoma, colon carcinoma, rectal carcinoma, brain carcinoma, liver carcinoma, breast carcinoma, ovary carcinoma, solid tumors, solid tumor metastases, angiofibromas, retrolental fibroplasia, hemangiomas, Karposi's

15   sarcoma, head and neck carcinomas, and combinations thereof.

The present method can be employed to suppress the growth of a tumor in any organism. In some embodiments, the subject is a mammal. In some embodiments, the mammal is a human.

The presently disclosed subject matter also provides a method for

20   identifying an antibody that specifically binds to an EGFR heterodimer. In some embodiments, the method comprises (a) isolating a membrane fraction of a cell, wherein the membrane fraction of the cell comprises EGFR and at least one of ERBB2, ERBB3, and ERBB4; (b) immunizing a mammalian subject with the membrane fraction; and (c) purifying an antibody from the

25   antisera that specifically binds to an EGFR heterodimer. In some embodiments, (i) the cell is a mammalian cell that does not normally express any member of the ERBB family; and (ii) the mammalian cell has been transformed with one or more expression constructs that encode one or more ERBB family members selected from the group consisting of EGFR,

30   ERBB2, ERBB3, and ERBB4, wherein the transforming results in the mammalian cell expressing EGFR and at least one of ERBB2, ERBB3, and ERBB4 in a membrane of the cell. In some embodiments, the present method further comprises isolating spleen cells from the mouse immunized

with the membrane fraction; generating hybridomas using the spleen cells; and identifying a hybridoma that produces a monoclonal antibody that specifically binds to an EGFR heterodimer.

The presently disclosed subject matter also provides a method for
5    identifying a compound that inhibits formation of a heterodimer between EGFR and another ERBB family member. In some embodiments, the method comprises (a) producing a first solution comprising a plurality of molecules that bind to EGFR; (b) producing a second solution comprising a plurality of molecules that bind to the other ERBB family member; (c)
10   contacting the first solution with a first structure comprising a plurality of EGFR homodimers under conditions sufficient to allow any of the plurality of molecules to bind to the EGFR homodimers; (d) contacting the second solution with a second structure comprising a plurality of homodimers of the other ERBB family member under conditions sufficient to allow any of the
15   plurality of molecules to bind to the homodimers of the other ERBB family member; (e) pooling any unbound compounds from the first and second solutions to produce a third solution; and (f) testing the unbound compounds in the third solution for an ability to inhibit formation of a heterodimer between EGFR and the other ERBB family member, whereby a compound
20   that inhibits formation of a heterodimer between EGFR and the other ERBB family member is identified. In some embodiments, the first solution and the second solution comprise polyclonal antisera produced by immunizing an animal with purified EGFR, the other ERBB family member, or combinations thereof. In some embodiments, the first solution and the second solution
25   comprise pooled monoclonal antibodies produced by hybridomas generated from an animal that had been immunized with purified EGFR, the other ERBB family member, or combinations thereof. In some embodiments, the first solution and the second solution are the same.

In some embodiments of the present method, the EGFR is human
30   EGFR and the other ERBB family member is human ERBB2.

In some embodiments of the present method, the first structure and the second structure each comprises a cell that has been engineered to express either EGFR or the other ERBB family member.

Accordingly, it is an object of the presently disclosed subject matter to provide a method for screening modulators for an ability to specifically bind to an EGFR heterodimer. This and other objects are achieved in whole or in part by the presently disclosed subject matter.

5      An object of the presently disclosed subject matter having been stated above, other objects and advantages of the presently disclosed subject matter will become apparent to those of ordinary skill in the art after a study of the following description and non-limiting Examples.


10                          Brief Description of the Drawings

Figures 1A and 1B disclose the sequence and structure of the Wa5 mutation.

In Figure 1A, the top panel depicts DNA and amino acid sequence comparisons of the $Egfr^{Wa5}$ (Wa5) kinase region containing the ENU-induced

15     mutation with wild type Egfr (+). The bottom panel depicts the amino acid sequence similarity between highly divergent human Ser/Thr and Tyr kinases. All DFG-containing kinases listed have a functional kinase, whereas those lacking the DFG motif (shown below the dotted line) are kinase dead.

20     Figure 1B depicts a ribbon structure of the human EGFR kinase domain (Protein Data Bank Identification No. 1M14) displayed with RasMol showing positions of amino acids altered in different Egfr mutations. Lys723 and Asp815 have been proven to be essential for kinase activity in vitro. Sites of existing Egfr mutations in mice (and their alleles) are indicated. The

25     amino acid numbering is based upon the mature mouse Egfr sequence (i.e., the protein without the 24 amino acid signal sequence).

Figures 2A and 2B depict the effect of $Egfr^{Wa5}$ on $Apc^{Min}$-mediated intestinal tumorigenesis.

Figure 2A depicts the number of polyps in $Apc^{Min}$/+ mice with and

30     without an $Egfr^{Wa5}$ allele. Each dot represents the total number of polyps (>0.3 mm in diameter) from individual three-month-old mice. Horizontal lines are means for each genotype. n = 20 for Egfr wild type and n = 22 for $Egfr^{Wa5}$/+.

Figure 2B depicts the sizes of polyps (diameter) from the tumor-bearing mice summarized in Figure 2A. Each dot represents the mean size of all polyps from a single mouse. Horizontal lines are means for each group.

5        Figures 3A and 3B depict EGF-induced tyrosine phosphorylation *in vivo*.

Figure 3A depicts EGF-induced tyrosine phosphorylation *in vivo* in liver and skin extracts from two-day-old pups. Figure 3B depicts EGF-induced tyrosine phosphorylation *in vivo* in liver extract from seven-day-old

10    pups. Wild type *Egfr* (+/+) and *Egfr$^{Wa5}$/+* (Wa5/+) pups were injected subcutaneously with either PBS (P) or EGF ($E_{0.5}$, $E_{1.0}$, and $E_{10}$ refer to 0.5, 1.0, and 10 µg/g of body weight) before euthanizing 10 minutes later. Extracts were prepared and equal amounts (15 µg) were analyzed by western blot analysis using either anti-phophotyrosine antibody (pY) or anti-

15    EGFR antibody (EGFR). kD, molecular weight markers; arrowheads, EGFR; arrows, p120 and p55 phosphorylation targets.

Figures 4A-4E depict western blot analysis of extracts from CHO cells transiently transfected with wild type and mutant *EGFR* expression vectors.

Figure 4A depicts levels of total EGFR (EGFR) and phosphorylated

20    EGFR (pY) protein in extracts from wild type *EGFR* (Wt), *EGFR$^{kd}$* (a kinase-dead EGFR; kd; Honegger *et al.*, 1987), or *EGFR$^{Wa5}$* transfected CHO cells. Cells were transfected with 0.1, 0.2, or 0.5 µg of the respective expression vector. Figure 4B depicts dimerization complexes of Wt, kd, and Wa5 in extracts from CHO cells transiently transfected with 0.5 µg of respective

25    expression vector followed by chemical cross-linking. Arrowheads, monomers; arrows, dimers. Figure 4C depicts levels of total and phosphorylated EGFR in extracts from CHO cells transiently co-transfected with 0.2 µg of wild type *EGFR* expression vector and 0.1 or 0.2 µg of Wt, kd, or Wa5 *EGFR* expression vectors. Figure 4D depicts immunoprecipitation-

30    western blot analysis of extracts from CHO cells transiently co-transfected with wild type *EGFR* expression vector and FLAG-tagged (f) Wt, kd, or Wa5 *EGFR* expression vectors. After immunoprecipitation with anti-FLAG antibody, immune complexes were divided equally before electrophoresis

and blotting to three different membranes for detection of EGFR, FLAG, and pY. Figure 4E depicts levels of total and phosphorylated EGFR in extracts from CHO cells transiently co-transfected with 0.25 µg of $EGFR^{Dsk5}$ expression vector and 0.05, 0.1, or 0.25 µg of $EGFR^{Wa5}$ expression vector or

5    vice versa. C, extracts from non-transfected CHO cells; EGFR, anti-EGFR antibody; pY, anti-phosphotyrosine antibody; FLAG, anti-FLAG antibody; triangles, dilution series of co-transfected constructs.

Figures 5A and 5B depict western blot analysis of extracts from CHO cells transiently transfected with *ERBB2* and mutant *EGFR* expression

10   vectors.

Figure 5A depicts levels of total EGFR (EGFR), total ERBB2 (B2) and phosphorylated EGFR or ERBB2 (pY) in extracts from cells co-transfected with 0.1 µg *ERBB2* (B2) expression vector alone or with 0.1, 0.3, or 0.5 µg of wild type *EGFR* (Wt), $EGFR^{kd}$ (kd), or $EGFR^{Wa5}$ (Wa5) expression vectors.

15   Figure 5B depicts levels of immunoprecipitated (IP) EGFR, ERBB2, and phosphorylated EGFR or ERBB2 in extracts from cells co-transfected with *ERBB2* expression vector and wild type *EGFR*, $EGFR^{kd}$, or $EGFR^{Wa5}$ expression vectors as described for Figure 5A. Only 0.3 and 0.5 µg of *EGFR* expression vectors are shown. After immunoprecipitation with anti-

20   EGFR or anti-ERBB2 antibodies, the immune complexes were divided equally into three aliquots before electrophoresis and blotting to three different membranes for detection of EGFR, ERBB2, and pY. Triangles, dilution series of co-transfected constructs.

Figures 6A and 6B depict a model for Wa5 antimorphic action.

25   In Figure 6A, EGFR monomers form ligand independent pre-dimers and, in one model, irreversible EGFR-Wa5 homodimers are preferentially formed. Upon ligand binding, dimers become stabilized and internalized, although they do not posses a fully activated kinase. When EGFR-Wa5 or Wa5-Wa5 homodimers form, they remain as inactive kinases. However,

30   inter-dimer interactions might play major role in fully activating the kinases of wild type receptor. Only one out of 16 combinations in this model will have EGFR dimer-dimer interactions, thus resulting in a dramatic reduction in overall ligand-induced phosphorylation when an equal numbers of EGFR

and Wa5 receptors exist. In Figure 6B, unlike EGFR, which is predominately on the cell surface, ERBB2 is primarily in cytoplasmic vesicles. Similar to the homodimers, ligand-stabilized EGFR dimers get internalized and can then form hetero-tetrameric interactions with ERBB2. Since the kinase of
5    ERBB2 has much greater activity than EGFR, complexes with Wa5 do not inhibit phosphorylation. Dark grey, black, and light grey lines indicate EGFR, Wa5, and ERBB2 receptors, respectively. Single lines, monomers; double lines, dimers; circles, ligand; black ovals, inactive tyrosine kinases; light grey explosions, active kinases; P, phosphorylation sites.
10

## Brief Description of the Sequence Listing

SEQ ID NOs: 1 and 2 present the nucleotide and amino acid sequences of human EGFR corresponding to GENBANK® Accession Numbers NM_005228 and NP_005219, respectively.
15    SEQ ID NOs: 3 and 4 present the nucleotide and amino acid sequences of mouse EGFR corresponding to GENBANK® Accession Numbers NM_207655 and NP_997538, respectively.

SEQ ID NOs: 5 and 6 present the nucleotide and amino acid sequences of the mouse EGFR$^{Wa5}$, respectively. SEQ ID NO: 5 differs from
20    SEQ ID NOs: 3 by the substitution of a G for an A at nucleotide 2850, resulting in a substitution of Gly for Asp at amino acid 857. Note that because EGFR has a 24 amino acid signal sequence, Asp857 in SEQ ID NOs: 2, 4, or 6 is Asp833 as referred to herein.

SEQ ID NOs: 7-9 are the sequences of oligonucleotide primers that
25    are used in polymerase chain reaction amplifications of the wild type and Wa5 alleles. SEQ ID NOs: 7 and 9 together can be used to amplify the wild type allele, and SEQ ID NOs: 8 and 9 can be used to amplify the Wa5 allele.

SEQ ID NOs: 10-21 are the nucleic acid and amino acid sequences of human and murine ERBB2, 3, and 4 as found int eh GENBANK® database
30    under the following Accession Nos.:

|        | Human | | Mouse | |
|--------|-------|-------|-------|-------|
|        | Nucleic Acid | Amino Acid | Nucleic Acid | Amino Acid |
| ERBB2 | NM_004448; SEQ ID NO: 10 | NP_004439.2; SEQ ID NO: 11 | NM_001003817; SEQ ID NO: 12 | NP_001003817.1; SEQ ID NO: 13 |
| ERBB3 | NM_001982; SEQ ID NO: 14 | NP_001973.2; SEQ ID NO: 15 | AY686636.1; SEQ ID NO: 16 | AAT95433.1; SEQ ID NO: 17 |
| ERBB4 | NM_005235; SEQ ID NO: 18 | NP_005226.1; SEQ ID NO: 19 | XM_136682; SEQ ID NO: 20 | XP_136682.4; SEQ ID NO: 21 |

## Detailed Description

As disclosed herein, the $Egfr^{Wa5}$ mutation is a kinase dead antimorphic allele of $Egfr$ that abolishes kinase activity of Egfr homodimers.

5  However, $Wa5$ does not affect kinase activity in Egfr/ERBB2 heterodimers. Thus, there is a qualitative difference in the way that an Egfr molecule interacts with other Egfr molecules to form homodimers as compares with the interaction between Egfr and ERBB2 during the formation of heterodimers. This qualitative difference can be exploited to specifically

10  inhibit the activity of heterodimers of EGFR with other members of the ERBB family.

### I.    Definitions

All technical and scientific terms used herein, unless otherwise defined below, are intended to have the same meaning as commonly understood by one of ordinary skill in the art, and references to techniques

15  understood by one of ordinary skill in the art, and references to techniques employed herein are intended to refer to the techniques as commonly understood in the art, including variations on those techniques or substitutions of equivalent techniques which would be apparent to one of skill in the art. While the following terms are believed to be well understood

20  by one of ordinary skill in the art, the following definitions are set forth to facilitate explanation of the presently disclosed subject matter.

Following long-standing patent law convention, the terms "a", "an", and "the" refer to "one or more" when used in this application, including in the claims.

As used herein, unless specifically indicated otherwise, the word "or"
5    is used in the "inclusive" sense of "and/or" and not the "exclusive" sense of "either/or".

As used herein, the term "ERBB family member" refers to a nucleic acid encoding a member of the ERBB family including, but not limited to ERBB1/HER1/EGFR,     ERBB2/HER2/NEU,     ERBB3/HER3,     and
10   ERBB4/HER4, or a polypeptide encoded by such a nucleic acid. These genes and gene products are found in a diversity of species. In some embodiments, the ERBB family members are mammalian ERBB family members. Representative mammals include human, mice, and rats. The nucleic acid and amino acid sequences for numerous ERBB family members
15   are present in publicly available databases (e.g. GENBANK®, available from the website of the National Center for Biotechnology Information (NCBI)), and include the nucleic acid and amino acid sequences presented in SEQ ID NOs: 1-6 and 10-21. With regard to particular members of the ERBB family and as indicated above, each member of the family is known by at least two
20   different names. For example, ERBB1, HER1, and EGFR all refer to the same gene and/or gene product, as do ERBB2, HER2, and NEU.

Additionally, consistent with usage in the art, identifications of genes or gene products that are presented in all capital letters refer to human genes and/or gene products or are referring to a family member without
25   reference to the species from which it is derived. For genes and gene products from murine sources (e.g., mice), the first letter is capitalized and other letters are presented in lower case. Also typically, references to genes are presented in italics, and references to polypeptides are presented in normal type. Thus, EGFR refers to either a human EGFR gene or to an
30   EGFR gene generally (i.e., without reference to a particular species). Similarly, EGFR refers to a human EGFR polypeptide, or to an EGFR polypeptide without reference to a particular species of origin. Egfr refers to a mouse Egfr gene, and Egfr refers to a mouse Egfr polypeptide.

Furthermore, different alleles of the ERBB family can be represented in superscript form (*e.g.* *Egfr*$^{Wa5}$ or *EGFR*$^{kd}$) or, in the absence of specific reference to the ERBB family member when the specific ERBB family member is clear, in normal sized type (*e.g.*, *Wa5* or *kd*). The absence of a

5      specific superscripted allele name indicates that the allele is a wild type allele or that the gene is being referred to generally without reference to a specific allele. The same italicization rules apply when a specific allele is identified.

As used herein, the terms "ligand" and "binding molecule", and

10     grammatical variants thereof, refer to a molecule or other chemical entity having a capacity for binding to a target. A ligand can comprise a peptide, an oligomer, a small molecule (*e.g.*, a chemical compound), an antibody or fragment thereof, and/or any other affinity agent. In some embodiments, a ligand is a natural ligand of an ERBB family member such as EGF, TGFα,

15     betacellulin, heparin-binding-EGF, epiregulin, or any other natural ligand for an ERBB family member. In some embodiments, a ligand is an artificial ligand such as an antibody or a small molecule that binds to an EGFR heterodimer. In some embodiments, the phrase "binding molecules" refers to molecules (*e.g.* antibodies or small molecules) that modulate the activity

20     of EGFR heterodimers. In some embodiments, a binding molecule prevents the formation of EGFR heterodimers. In some embodiments, a binding molecule prevents signal transduction via an EGFR heterodimer.

As used herein, the phrase "EGFR heterodimer" refers to a heterodimer between EGFR/ERBB1/HER1 and another member of the

25     ERBB family. Unless otherwise indicated, the term "heterodimer" also refers to a heterodimer containing EGFR/ERBB1/HER1 and another ERBB family member. Exemplary heterodimers include heterodimers of EGFR and ERBB2, of EGFR and ERBB3, and of EGFR and ERBB4.

Additionally, the terms "heterodimer" and "homodimer" refer not only

30     to interactions of two molecules (*i.e.* to form dimers), but also to higher order interactions of more than two molecules. Thus, unless otherwise indicated, the term "heterodimer" is intended to encompass interactions of, for example, three, four, five, six, seven, eight or more members of the ERBB

family, wherein at least two of the interacting molecules are different family members.    Similarly, the term "homodimer" is intended to encompass interactions of, for example, three, four, five, six, seven, eight or more members of the ERBB family, wherein all of the interacting molecules are the

5    same.

As used herein, the term "modulate", and grammatical variants thereof, refers to an increase, decrease, or other alteration of any or all biological activities or properties of an ERBB family member.  Similarly, the term "modulator" refers to a compound (*e.g.* a antibody, antibody derivative,

10    or small molecule) that in some embodiments inhibits the formation of a heterodimer of EGFR with another ERBB family member, thereby modulating signal transduction through the heterodimer, or modulates any or all biological activities or properties of a heterodimer of EGFR with another family member by interacting (*e.g.* specifically binding to) the heterodimer.

15         The term "small molecule" as used herein refers to a compound, for example an organic compound, with a molecular weight in some embodiments of less than about 1,000 daltons, in some embodiments less than about 750 daltons, in some embodiments less than about 600 daltons, and in some embodiments less than about 500 daltons.  A small molecule

20    also has a computed log octanol-water partition coefficient that in some embodiments is in the range of about -4 to about +14, and in some embodiments is in the range of about -2 to about +7.5.

The term "binding" refers to an affinity between two molecules, for example, a ligand (*e.g.*, a ligand of an ERBB family member) and a target

25    (*e.g.*, an ERBB family member).  In some embodiments, the term "binding" refers to a specific binding of one molecule for another in a mixture of molecules.  The binding of a ligand to a target molecule can be considered specific if the binding affinity is about $1 \times 10^4$ M$^{-1}$ to about $1 \times 10^6$ M$^{-1}$ or greater.  For example, the binding of an antibody to an antigen can be

30    thought of as having at least two components: an affinity, which refers to the strength at which the antibody binds an antigen, and a specificity, which refers to the level of cross-reactivity an antibody displays between closely related antigens.

The phrase "specifically (or selectively) binds", when referring to the binding capacity of a ligand, refers to a binding reaction which is determinative of the presence of the protein in a heterogeneous population of proteins and other biological materials. In some embodiments, the

5        binding of a modulator is considered to be specific, that is the modulator binds to an EGFR heterodimer but does not appreciably bind to either EGFR homodimers or homodimers of other ERBB family members. As disclosed herein, the interaction of EGFR with other ERBB molecules is qualitatively different than interactions among EGFR molecules or among ERBB (non-

10       EGFR) molecules.

The phases "substantially lack binding" or "substantially no binding", as used herein to describe binding of a ligand in a control tissue, refers to a level of binding that encompasses non-specific or background binding, but does not include specific binding.

15       The term "about", as used herein when referring to a measurable value such as an amount of weight, time, dose, etc., is meant to encompass variations of in some embodiments ±20%, in some embodiments ±10%, in some embodiments ±5%, in some embodiments ±1%, and in some embodiments ±0.1% from the specified amount, as such variations are

20       appropriate to perform the disclosed methods.

II.      Identification of Ligands that Bind to EGFR Heterodimers

In some embodiments, the presently disclosed subject matter provides methods for identifying ligands (*e.g.* antibodies or small molecules) that specifically bind to EGFR/ERBB heterodimers (*e.g.* heterodimers of

25       EGFR and another ERBB family member) and modulate the activity of the EGFR/ERBB heterodimer. In some embodiments, the presently disclosed subject matter provides methods for identifying ligands (*e.g.* antibodies or small molecules) that prevent the heterodimerization of two different ERBB family members by binding to one or the other. In some embodiments, the

30       method comprises (a) contacting a first structure comprising an EGFR/ERBB heterodimer with a first solution, the first solution comprising the plurality of compounds; (b) removing any compounds bound to the first structure to produce a second solution; (c) contacting a second structure comprising an

EGFR homodimer with the second solution, wherein the first structure and the second structure are identical except that the second structure does not contain an ERBB family member other than EGFR; (d) recovering any unbound compounds to produce a third solution, whereby a compound that
5    binds to a heterodimer of EGFR and another ERBB family member is identified.

In some embodiments, the method further comprises (e) contacting a third structure comprising an EGFR/RBB heterodimer with the third solution; and (f) detecting binding of a compound to the EGFR/ERBB heterodimer on
10   the third structure. In some embodiments, the method further comprises negatively selecting the plurality of compounds by contacting the plurality of compounds with a structure that is identical to the first and second structures except that it does not contain any ERBB family members. Exemplary structures are cells and cell components (e.g. membrane fractions), although
15   other structures such as solid supports can also be employed with the methods disclosed herein. Thus, if the structures are cells, negative selection can be performed on a cell that does not express any ERBB family members. Additional negative selection steps can including contacting a cell that contains only homodimers of ERBB family members. The negative
20   selections step(s) can be performed before or after contacting the structure comprising the EGFR heterodimer with the plurality of potential binding compounds. For example, 32D cells which are devoid of all EGFR and ERBB molecules and do not express any of their cognate ligands can be used to express EGFR and ERBB molecules, either individually or in
25   combinations, to screen for interactions as described above. Exogenous ligands can be added to screen for receptor interactions that are specific for EGFR/ERBB heterodimers. In particular, betacellulin, heparin-binding-EGF, and epiregulin can be used since they are reported to be selective for EGFR/ERBB heterodimer interactions. Likewise, COS cells, which are also
30   devoid of EGFR and ERBB receptors but which express endogenous ligands, can be used to distinguish unique EGFR/ERBB heterodimers.

As described hereinabove, the $Egfr^{Wa5}$ mutation is a kinase dead antimorphic allele of Egfr that abolishes kinase activity of Egfr homodimers

but does not affect kinase activity in Egfr/ERBB2 heterodimers. Thus, there is a qualitative difference in the way that an Egfr molecule interacts with other Egfr molecules to form homodimers as compares with the interaction between Egfr and ERBB2 during the formation of heterodimers. This

5   qualitative difference can be exploited to specifically inhibit the activity of heterodimers of EGFR with other members of the ERBB family.

As used herein, the terms "first structure", "second structure", and "third structure" refer to any structure that comprises one or more ERBB family member polypeptides and allows the one or more ERBB family

10   member polypeptides to form homodimers and/or heterodimers between the one or more ERBB family member polypeptides in the absence of an EGFR heterodimer binding molecule as disclosed herein. Representative structures include cells and components of cells, including for example, membrane fractions from cells.

15   .   In some embodiments, the first, second, and third structures are cells that either normally express one or more ERBB family member polypeptides or have been engineered to express one or more ERBB family member polypeptides. Methods for producing expression vectors that encode members of the ERBB family and for transforming mammalian cells with

20   these vectors to express the one or more ERBB family members in the transformed cells are known in the art.

In some embodiments, ligands that specifically bind to EGFR heterodimers are isolated from a library of potential ligands. As used herein, the term "library" means a collection of molecules. A library can contain a

25   few or a large number of different molecules, varying from at least two molecules to several billion molecules or more. A molecule can comprise a naturally occurring molecule, or a synthetic molecule that is not found in nature. Optionally, a plurality of different libraries can be employed simultaneously for screening.

30   Representative libraries include but are not limited to a peptide library (U.S. Patent Nos. 6,156,511, 6,107,059, 5,922,545, and 5,223,409), an oligomer library (U.S. Patent Nos. 5,650,489 and 5,858,670), an aptamer library (U.S. Patent No. 6,180,348 and 5,756,291), a small molecule library

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(U.S. Patent Nos. 6,168,912 and 5,738,996), a library of antibodies or antibody fragments (for example, an scFv library or an Fab antibody library; U.S. Patent Nos. 6,174,708, 6,057,098, 5,922,545, 5,840,479, 5,780,225, 5,702,892, and 5,667,988), a library of nucleic acid-protein fusions (U.S.

5      Patent No. 6,214,553), and a library of any other affinity agent that can potentially bind to an irradiated tumor (*e.g.*, U.S. Patent Nos. 5,948,635, 5,747,334, and 5,498,538). In some embodiments, a library is a phage-displayed antibody library. In some embodiments, a library is a phage-displayed scFv library. In some embodiments, a library is a phage-displayed

10     Fab library. In some embodiments, a library is a soluble scFv antibody library.

The molecules of a library can be produced *in vitro*, or they can be synthesized *in vivo*, for example by expression of a molecule *in vivo*. Also, the molecules of a library can be displayed on any relevant support, for

15     example, on bacterial pili (Lu *et al.*, 1995) or on phage (Smith, 1985).

A library can comprise a random collection of molecules. Alternatively, a library can comprise a collection of molecules having a bias for a particular sequence, structure, conformation, or in the case of an antibody library, can be biased in favor of antibodies that bind to a particular

20     antigen or antigens (for example, an EGFR heterodimer). *See e.g.*, U.S. Patent Nos. 5,264,563 and 5,824,483. Methods for preparing libraries containing diverse populations of various types of molecules are known in the art, for example as described in U.S. Patents cited herein above. Numerous libraries are also commercially available.

25     II.A.   Peptide Libraries

In some embodiments, a peptide library comprises peptides comprising three or more amino acids, in some embodiments at least five, six, seven, or eight amino acids, in some embodiments up to 50 amino acids or 100 amino acids, and in some embodiments up to about 200 to 300

30     amino acids.

The peptides can be linear, branched, or cyclic, and can include non-peptidyl moieties. The peptides can comprise naturally occurring amino

acids, synthetic amino acids, genetically encoded amino acids, non-genetically encoded amino acids, and combinations thereof.

A biased peptide library can also be used, a biased library comprising peptides wherein one or more (but not all) residues of the peptides are

5    constant.  For example, an internal residue can be constant, so that the peptide sequence is represented as:

$$(XAA_1)_m - (AA)_1 - (XAA_2)_n$$

where $XAA_1$ and $XAA_2$ are any amino acid, wherein $XAA_1$ and $XAA_2$ are the same or different amino acids, m and n indicate a number XAA residues,

10   wherein m and n are independently chosen from the range of 2 residues to 20 residues in some embodiments, and from the range of 4 residues to 9 residues in some embodiments, and AA is the same amino acid for all peptides in the library.  In some embodiments, AA is located at or near the center of the peptide.  More specifically, in some embodiments m and n are

15   not different by more than 2 residues, and in some embodiments m and n are equal.

In some embodiments, a library is employed in which AA is tryptophan, proline, or tyrosine.  In some embodiments, AA is phenylalanine, histidine, arginine, aspartate, leucine, or isoleucine.  In some embodiments,

20   AA is asparagine, serine, alanine, or methionine.  In some embodiments, AA is cysteine or glycine.

A biased library used for screening also includes a library comprising molecules previously selected by other screening methods.

In some embodiments of the presently disclosed subject matter, the

25   method for screening is performed using a phage peptide library.  Phage display is a method to discover peptide ligands while minimizing and optimizing the structure and function of proteins.  Phage are used as a scaffold to display recombinant libraries of peptides and provide a vehicle to recover and amplify the peptides that bind to antigens *in vivo* or *in vitro*.

30   The T7 phage has an icosahedral capsid made of 415 proteins encoded by gene 10 during its lytic phase.  The T7 phage display system has the capacity to display peptides up to 15 amino acids in size at a high copy number (415 per phage).  Unlike filamentous phage display systems,

peptides displayed on the surface of T7 phage are not capable of peptide secretion. T7 phage also replicate more rapidly and are extremely robust when compared to other phage. The stability allows for bioscreening selection procedures that require persistent phage infectivity. Accordingly,

5    the use of T7-based phage display is an aspect of some embodiments of the presently disclosed subject matter. Example 9 describes a representative method for preparation of a T7 phage peptide library that can be used to perform the screening methods disclosed herein.

A phage peptide library to be used in accordance with the screening

10    methods of the presently disclosed subject matter can also be constructed in a filamentous phage, for example M13 or an M13-derived phage. In some embodiments, the encoded antibodies are displayed at the exterior surface of the phage, for example by fusion to the product of M13 gene III. Methods for preparing M13 libraries can be found in Sambrook & Russell, 2001,

15    among other places.

II.B.    Phage Antibody Libraries

In some embodiments, a ligand that binds to an EGFR heterodimer is an antibody, or a fragment thereof. To identify antibodies and fragments thereof that bind to EGFR heterodimers, libraries can be screened using the

20    methods disclosed herein. Libraries that can be screened using the disclosed methods include, but are not limited to libraries of phage-displayed antibodies and antibody fragments, and libraries of soluble antibodies and antibody fragments.

"Fv" is the minimum antibody fragment that contains a complete

25    antigen recognition and binding site. In a two-chain Fv species, this region consists of a dimer of one heavy and one light chain variable domain in tight, non-covalent association. In a single-chain Fv species (scFv), one heavy and one light chain variable domain can be covalently linked by a flexible peptide linker such that the light and heavy chains can associate in a

30    "dimeric" structure analogous to that in a two-chain Fv species. It is in this configuration that the three complementarity-determining regions (CDRs) of each variable domain interact to define an antigen-binding site on the surface of the $V_H$-$V_L$ dimer. Collectively, the six CDRs confer

antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site. For a review of scFv, see Pluckthun, 1994.

5         The term "antibodies and fragments thereof", and grammatical variations thereof, refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules; i.e., molecules that contain an antigen-binding site that specifically bind an antigen. As such, the term refers to immunoglobulin proteins, or functional portions thereof, including

10      polyclonal antibodies, monoclonal antibodies, chimeric antibodies, hybrid antibodies, single chain antibodies (e.g., a single chain antibody represented in a phage library), mutagenized antibodies, humanized antibodies, and antibody fragments that comprise an antigen binding site (e.g., Fab and Fv antibody fragments). Thus, "antibodies and fragments thereof" include, but

15      are not limited to monoclonal, chimeric, recombinant, synthetic, semi-synthetic, or chemically modified intact antibodies having for example Fv, Fab, scFv, or $F(ab)_2$ fragments.

        The immunoglobulin molecules of the presently disclosed subject matter can be of any type (e.g., IgG, IgE, IgM, IgD, IgA and IgY), class (e.g.,

20      IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2), or subclass of immunoglobulin molecule. In some embodiments, the antibodies are human antigen-binding antibody fragments and include, but are not limited to, Fab, Fab' and F(ab')$_2$, Fd, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv), and fragments comprising either a $V_L$ or $V_H$ domain. Antigen-binding

25      antibody fragments, including single-chain antibodies, can comprise the variable region(s) alone or in combination with the entirety or a portion of the following: hinge region, CH1, CH2, and CH3 domains. Also included in the presently disclosed subject matter are antigen-binding fragments comprising any combination of variable region(s) with a hinge region, CH1, CH2, and

30      CH3 domains.

        The antibodies and fragments thereof of the presently disclosed subject matter can be from any animal origin including birds and mammals. For example, the antibodies can be human, murine (e.g., mouse and rat),

donkey, ship rabbit, goat, guinea pig, camel, horse, or chicken. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries or from animals transgenic for one or more

5    human immunoglobulin and that do not express endogenous immunoglobulins, as described, for example in, U.S. Patent No. 5,939,598.

In some embodiments of the presently disclosed subject matter, an antibody library (for example, a library of scFv antibodies) can be used to perform the disclosed screening methods. In these embodiments, a ligand

10   that binds to an EGFR heterodimer is an antibody or a fragment thereof that binds an EGFR heterodimer. Antibodies that bind EGFR heterodimers can be identified by screening a phage antibody library. Similarly, antibodies that prevent the formation of EGFR heterodimers can be identified by screening a phage antibody library. Such a library can be constructed, for example, in

15   M13 or an M13-derived phage. See e.g., U.S. Patent Nos. 6,593,081; 6,225,447; 5,580,717; and 5,702,892, all incorporated by reference herein.

Phage-displayed recombinant antibodies are genetically cloned and expressed on the tip of the M13 bacteriophage (McCafferty et al., 1990). M13 phage infects E. coli that carry an F' episome (plasmid) and constantly

20   produce and secrete intact M13 virus particles without lysing the host cell. The components of the M13 phage include phage DNA, coat proteins, gene III attachment proteins, and other proteins that are fused to the phage proteins. There are 3-5 copies of the gene III attachment proteins located on the exterior of the phage that are responsible for phage attachment to

25   receptors on E. coli cells.

In some embodiments, M13 phage-displayed recombinant antibodies can be created by linking DNA from antibody-producing B lymphocytes to the phage gene III DNA using the pCANTAB vector (see Example 10). The proteins encoded by the antibody in gene III DNA are fused to one another

30   to produce an antibody-gene III fusion protein. A bacteriophage carrying the gene fusion will simultaneously contain the antibody DNA and express an antibody molecule on the gene III protein.

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A representative, non-limiting approach to obtain and characterize antigen-specific recombinant antibodies or antibody fragments (for example, scFv antibodies or human Fab antibodies) is as follows. Phage antibody selections can be performed using antigens (e.g. isolated EGFR heterodimers) immobilized on solid supports or biotinylated antigens and streptavidin magnetic beads. An aliquot of a phage antibody library can be applied to a structure comprising the target. Nonspecific phage antibodies are thereafter washed off of the structure comprising the target, and phage that encode bound antibodies can be eluted and used to infect E. coli. Infected E. coli can be plated and rescued with helper phage to produce an target-enriched phage antibody library. The target-enriched library (i.e., a library pre-selected for binding to a particular antigen of interest) can be used in a second round of selection for binding to the structure comprising the target. Subsequent rounds of selection on targets and helper phage rescue can be used until the desired target-specific antibodies are obtained. Colonies stemming for phage antibody selections can be picked from agar plates manually or by using a colony picker (for example, the QPix2 Colony Picker from Genetix USA Inc., Boston, Massachusetts, United States of America). Picked colonies can then be transferred to appropriate vessels, for example microwell plates, and can be used to produce soluble recombinant antibodies. See e.g., Example 10.

Phage-displayed recombinant antibodies have several advantages over polyclonal antibodies or hybridoma-derived monoclonal antibodies. Phage-displayed antibodies can be generated within 8 days. Recombinant antibody clones can be easily selected by panning a population of phage-displayed antibodies against immobilized antigen (McCafferty et al., 1990). The antibody protein and antibody DNA are simultaneously contained in one phage particle (Better et al., 1988). Liters of phage-displayed recombinant antibodies can be produced inexpensively from bacterial culture supernatant and the phage antibodies can be used directly in immunoassays or other procedures without purification.

Phage display technology makes possible the direct isolation of monovalent scFv antibodies. The small size of scFv antibodies makes it the

antibody format of choice, for example, for tumor penetration and rapid clearance from the blood (Adams et al., 1995; Adams, 1998; Yokota et al., 1992).   The human phage antibody library can be used to develop antibodies suitable for clinical trials.  Human scFv. antibodies have entered clinical trials (Hoogenboom & Winter, 1992).  The human phage antibody library can be used to develop antibodies suitable for clinical trials.  Anti-melanoma antibodies have been developed using these phage libraries (Cai & Garen, 1995), as well as antibodies to antigens found in ovarian carcinoma (Figini et al., 1998).

Using a phage-displayed approach for the production of antibodies, scFv antibody clones that bind to EGFR heterodimers are identified as disclosed herein.   Fv regions are sequenced and bivalent functional reagents are designed and tested, for example using an assay as disclosed herein.   Thus, an exemplary, but non-limiting source for an antibody, or derivative or fragment thereof, is a recombinant phage-displayed antibody library.

The recombinant phage can comprise antibody-encoding nucleic acids isolated from any suitable vertebrate species, including in alternative embodiments mammalian species such as human, mouse, and rat.  Thus, in some embodiments the recombinant phage encode an antibody wherein both the variable and constant regions are encoded by nucleic acids isolated from the same species (for example, human, mouse, or rat).   In some embodiments, the recombinant phage encode chimeric antibodies, wherein the phrase "chimeric antibodies" (and grammatical variations thereof) refers to antibodies having variable and constant domain regions that are derived from different species.   For example, in some embodiments the chimeric antibodies are antibodies having murine variable domains and human constant domains.

The scFv antibodies of the presently disclosed subject matter also include humanized scFv antibodies.  Humanized forms of non-human (for example, murine) scFv antibodies are chimeric scFv antibodies that contain minimal sequence derived from non-human immunoglobulins.  Humanized scFv antibodies include human scFvs in which residues from a

complementarity-determining region (CDR) are encoded by a nucleic acid encoding a CDR of a non-human species such as mouse, having the desired specificity, affinity, and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human

5   residues. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the framework regions are those of a human immunoglobulin consensus sequence. The humanized

10  antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin (Jones et al., 1986; Riechmann et al., 1988; Presta, 1992). Thus, as used herein, the term "humanized" encompasses chimeric antibodies comprising a human constant region, including those antibodies wherein all of the residues are

15  encoded by a human nucleic acid (see e.g., Shalaby et al., 1992; Mocikat et al., 1994).

### II.C.   Antibody Ligands

When phage-displayed antibodies bind to an antigen, they can be affinity-purified using the antigen. These affinity-purified phage can then be

20  used to infect and introduce the antibody gene back into E. coli. The E. coli can then be grown and induced to express a soluble, non-phage-displayed, antigen-specific recombinant antibody. Phage display technology is especially useful for producing antibodies to antigens that are either poorly immunogenic, readily degraded, only transiently produced, and/or for which

25  monoclonal and/or polyclonal antibodies are difficult to obtain.

Epitopes formed by the heterodimerization of ERBB family members are a high priority target because they might not be accessible to antibodies or other binding molecules because the heterodimerization of two or more different ERBB family members might be transient, and/or the heterodimer

30  itself might be quickly endocytosed. Phage scFv antibodies, for example, can be developed to these proteins by use of phage-displayed antibody libraries. Negative selection of phage can be first performed on a control tissue, for example a tissue that expresses no ERBB family members or only

expresses one family member (thus eliminating the possibility that heterodimers can form). This can eliminate antibodies that nonspecifically bind to, for example, antigens unrelated to EGFR heterodimers (*e.g.*, other cellular components) or that bind to ERBB family homodimers. Unbound

5　　phage can then be recovered and incubated with purified EGFR heterodimers, for example, EGFR/ERBB2 heterodimers. High affinity phage can then be recovered, for example by use of washing at pH 1.

The term "isolated", as used in the context of a nucleic acid, peptide, or polypeptide, indicates that the nucleic acid or polypeptide exists apart

10　　from its native environment and is not a product of nature. An isolated nucleic acid or polypeptide can exist in a purified form or can exist in a non-native environment such as a transgenic host cell. In some embodiments of the presently disclosed subject matter, "isolated" refers to the purification of an scFv antibody from a target tissue to which it has bound.

15　　　　Nucleic Acids. The terms "nucleic acid molecule" or "nucleic acid" each refer to deoxyribonucleotides or ribonucleotides and polymers thereof in single-stranded or double-stranded. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar properties as the reference natural nucleic acid.

20　　The terms "nucleic acid molecule" or "nucleic acid" can also be used in place of "gene", "cDNA", or "mRNA". Nucleic acids can be synthesized, or can be derived from any biological source, including any organism.

The term "substantially identical", as used herein to describe a degree of similarity between nucleotide sequences, refers to two or more sequences

25　　that have in some embodiments at least about least 60%, in some embodiments at least about 70%, in some embodiments at least about 80%, in some embodiments about 90% to about 99%, in some embodiments about 95% to about 99%, and in some embodiments about 99% nucleotide identity, as measured using one of the following sequence comparison

30　　algorithms (described herein below) or by visual inspection. The substantial identity exists in some embodiments in nucleotide sequences of at least about 100 residues, in some embodiments in nucleotide sequences of at

least about 150 residues, and in some embodiments in nucleotide sequences comprising a full length coding sequence.

5    Thus, substantially identical sequences can comprise mutagenized sequences, including sequences comprising silent mutations, or variably synthesized sequences. A mutation or variant sequence can comprise a single base change.

Another indication that two nucleotide sequences are substantially identical is that the two molecules specifically or substantially hybridize to each other under stringent conditions. In the context of nucleic acid

10    hybridization, two nucleic acid sequences being compared can be designated a "probe" and a "target". A "probe" is a reference nucleic acid molecule, and a "target" is a test nucleic acid molecule, often found within a heterogeneous population of nucleic acid molecules. A "target sequence" is synonymous with a "test sequence".

15    The phrase "hybridizing specifically to" refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex nucleic acid mixture (e.g., total cellular DNA or RNA).

The phrase "hybridizing substantially to" refers to complementary

20    hybridization between a probe nucleic acid molecule and a target nucleic acid molecule and embraces minor mismatches that can be accommodated by reducing the stringency of the hybridization media to achieve the desired hybridization.

"Stringent hybridization conditions" and "stringent hybridization wash

25    conditions" in the context of nucleic acid hybridization experiments such as Southern and Northern blot analysis are both sequence- and environment-dependent. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, 1993. Generally, highly stringent hybridization and wash conditions are

30    selected to be about 5°C lower than the thermal melting point ($T_m$) for the specific sequence at a defined ionic strength and pH. Typically, under "stringent conditions" a probe will hybridize specifically to its target subsequence, but to no other sequences.

The $T_m$ is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Very stringent conditions are selected to be equal to the $T_m$ for a particular probe. An example of stringent hybridization conditions for Southern or Northern Blot analysis of complementary nucleic acids having more than about 100 complementary residues is overnight hybridization in 50% formamide with 1 mg of heparin at 42°C. An example of highly stringent wash conditions is 15 minutes in 0.1X SSC at 65°C. An example of stringent wash conditions is 15 minutes in 0.2X SSC buffer at 65°C. *See* Sambrook & Russell, 2001 for a description of SSC buffer. Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. An example of medium stringency wash conditions for a duplex of more than about 100 nucleotides, is 15 minutes in 1X SSC at 45°C. An example of low stringency wash for a duplex of more than about 100 nucleotides, is 15 minutes in 4X to 6X SSC at 40°C. For short probes (*e.g.*, about 10 to 50 nucleotides), stringent conditions typically involve salt concentrations of less than about 1M Na$^+$ ion, typically about 0.01 to 1M Na$^+$ ion concentration (or other salts) at pH 7.0-8.3, and the temperature is typically at least about 30°C. Stringent conditions can also be achieved with the addition of destabilizing agents such as formamide. In general, a signal to noise ratio of 2-fold (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization.

The following are examples of hybridization and wash conditions that can be used to identify nucleotide sequences that are substantially identical to reference nucleotide sequences of the presently disclosed subject matter. In some embodiments, a probe nucleotide sequence hybridizes to a target nucleotide sequence in 7% sodium dodecyl sulfate (SDS), 0.5M NaPO$_4$, 1 mM ethylenediamine tetraacetic acid (EDTA) at 50°C followed by washing in 2X SSC, 0.1% SDS at 50°C. In some embodiments, a probe and target sequence hybridize in 7% sodium dodecyl sulfate (SDS), 0.5M NaPO$_4$, 1 mM EDTA at 50°C followed by washing in 1X SSC, 0.1% SDS at 50°C. In some embodiments, a probe and target sequence hybridize in 7% sodium dodecyl sulfate (SDS), 0.5M NaPO$_4$, 1 mM EDTA at 50°C followed by

washing in 0.5X SSC, 0.1% SDS at 50°C. In some embodiments, a probe and target sequence hybridize in 7% sodium dodecyl sulfate (SDS), 0.5M NaPO$_4$, 1 mM EDTA at 50°C followed by washing in 0.1X SSC, 0.1% SDS at 50°C. In some embodiments, a probe and target sequence hybridize in 7% sodium dodecyl sulfate (SDS), 0.5M NaPO$_4$, 1 mM EDTA at 50°C followed by washing in 0.1X SSC, 0.1% SDS at 65°C.

A further indication that two nucleic acid sequences are substantially identical is that proteins encoded by the nucleic acids are substantially identical, share an overall three-dimensional structure, or are biologically functional equivalents. These terms are defined further herein below. Nucleic acid molecules that do not hybridize to each other under stringent conditions are still substantially identical if the corresponding proteins are substantially identical. This can occur, for example, when two nucleotide sequences are significantly degenerate as permitted by the genetic code.

The term "conservatively substituted variants" refers to nucleic acid sequences having degenerate codon substitutions wherein the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues. See Batzer et al., 1991; Ohtsuka et al., 1985; Rossolini et al., 1994.

The term "subsequence" refers to a sequence of nucleic acids that comprises a part of a longer nucleic acid sequence. An exemplary subsequence is a probe, described herein above, or a primer. The term "primer" as used herein refers to a contiguous sequence comprising in some embodiments about 8 or more deoxyribonucleotides or ribonucleotides, in some embodiments about 10-20 nucleotides, and in some embodiments about 20-30 nucleotides of a selected nucleic acid molecule. The primers of the presently disclosed subject matter encompass oligonucleotides of sufficient length and appropriate sequence so as to provide initiation of polymerization on a nucleic acid molecule of the presently disclosed subject matter.

The term "elongated sequence" refers to an addition of nucleotides (or other analogous molecules) incorporated into the nucleic acid. For example, a polymerase (e.g., a DNA polymerase) can add sequences at the 3'

terminus of the nucleic acid molecule. In addition, the nucleotide sequence can be combined with other DNA sequences, such as promoters, promoter regions, enhancers, polyadenylation signals, intronic sequences, additional restriction enzyme sites, multiple cloning sites, and other coding segments.

5      Nucleic acids of the presently disclosed subject matter can be cloned, synthesized, recombinantly altered, mutagenized, or combinations thereof. Standard recombinant DNA and molecular cloning techniques used to isolate nucleic acids are known in the art. Site-specific mutagenesis to create base pair changes, deletions, or small insertions are also known in

10    the art. *See e.g.*, Sambrook & Russell, 2001; Silhavy *et al.*, 1984; Glover & Hames, 1995; Ausubel, 1995.

Polypeptides. As used herein, the phrase "substantially identical" refers to a nucleic acid sequence having in some embodiments at least about 45%, in some embodiments at least about 50%, in some embodiments

15    at least about 60%, in some embodiments at least about 70%, in some embodiments at least about 80%, in some embodiments at least about 90%, in some embodiments at least about 95%, and in some embodiments at least about 99% sequence identity, when compared over the full length of the open reading frame of nucleic acid encoding a single chain antibody that

20    binds to an EGFR heterodimer or that binds to an ERBB family member polypeptide to prevent its forming a heterodimer with another ERBB family member. Methods for determining percent identity are defined herein below.

Substantially identical polypeptides also encompass two or more polypeptides sharing a conserved three-dimensional structure.

25    Computational methods can be used to compare structural representations, and structural models can be generated and easily tuned to identify similarities around important active sites or ligand binding sites. *See* Saqi *et al.*, 1999; Barton, 1998; Henikoff *et al.*, 2000; Huang *et al.*, 2000.

Substantially identical proteins also include proteins comprising an

30    amino acid sequence comprising amino acids that are functionally equivalent to amino acids of a single chain antibody that binds to an EGFR heterodimer or that binds to an ERBB family member polypeptide to prevent its forming a heterodimer with another ERBB family member. The term "functionally

equivalent" in the context of amino acid sequences is known in the art and is based on the relative similarity of the amino acid side-chain substituents. *See* Henikoff & Henikoff, 2000. Relevant factors for consideration include side-chain hydrophobicity, hydrophilicity, charge, and size. For example,

5 arginine, lysine, and histidine are all positively charged residues; that alanine, glycine, and serine are all of similar size; and that phenylalanine, tryptophan, and tyrosine all have a generally similar shape. By this analysis, described further herein below, arginine, lysine, and histidine; alanine, glycine, and serine; and phenylalanine, tryptophan, and tyrosine; are defined

10 herein as biologically functional equivalents.

In making biologically functional equivalent amino acid substitutions, the hydropathic index of amino acids can be considered. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics, these are: isoleucine (+4.5); valine (+4.2);

15 leucine (+3.8); phenylalanine (+2.8); cysteine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

20 The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is generally understood in the art (Kyte & Doolittle, 1982). It is known that certain amino acids can be substituted for other amino acids having a similar hydropathic index or score and still retain a similar biological activity. In some embodiments, amino

25 acids for which the hydropathic indices are within ±2 of the original value are substituted for each other. In some embodiments, amino acids for which the hydropathic indices are within ±1 of the original value are substituted for each other. And in some embodiments, amino acids for which the hydropathic indices are within ±0.5 of the original value are substituted for

30 each other in making changes based upon similar hydropathicity values.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Patent No. 4,554,101 describes that the greatest local average hydrophilicity of a

protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity and antigenicity, for example, with a biological property of the protein. It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a

5   biologically equivalent protein.

As detailed in U.S. Patent No. 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1);

10   alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

In some embodiments, amino acids for which the hydrophilic indices are within ±2 of the original value are substituted for each other. In some

15   embodiments, amino acids for which the hydrophilic indices are within ±1 of the original value are substituted for each other. And in some embodiments, amino acids for which the hydrophilic indices are within ±0.5 of the original value are substituted for each other in making changes based upon similar hydropathicity values.

20   The term "substantially identical" also encompasses polypeptides that are biologically functional equivalents. The term "functional", as used herein to describe polypeptides comprising antibody ligands, refers two or more antibodies that are immunoreactive with the same molecules. In some embodiments, the two or more antibodies specifically bind a same target

25   molecule and substantially lack binding to a control antigen.

Techniques for detecting antibody-target molecule complexes are known in the art and include but are not limited to centrifugation, affinity chromatography, and other immunochemical methods. See also Manson, 1992; Law, 1996.

30   The presently disclosed subject matter also provides functional fragments of an antibody polypeptide. Such functional portion need not comprise all or substantially all of the polypeptide, but should comprise that region of the polypeptide that binds to the heterodimer.

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Isolated polypeptides and recombinantly produced polypeptides can be purified and characterized using a variety of standard techniques that are known to the skilled artisan. *See e.g.*, Schröder & Lübke, 1965; Schneider & Eberle, 1993; Bodanszky, 1993; Ausubel, 1995.

5          <u>Nucleotide and Amino Acid Sequence Comparisons</u>. The terms "identical" or percent "identity" in the context of two or more nucleotide or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum

10      correspondence, as measured using one of the sequence comparison algorithms disclosed herein or by visual inspection.

The term "substantially identical" in regards to a nucleotide or polypeptide sequence means that a particular sequence varies from the sequence of a naturally occurring sequence by one or more deletions,

15      substitutions, or additions, the net effect of which is to retain biological activity of a gene, gene product, or sequence of interest.

For sequence comparison, typically one sequence acts as a reference sequence to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a

20      computer program, subsequence coordinates are designated if necessary, and sequence algorithm program parameters are selected. The sequence comparison algorithm then calculates the percent sequence identity for the designated test sequence(s) relative to the reference sequence, based on the selected program parameters.

25      Optimal alignment of sequences for comparison can be conducted, for example, by the local homology algorithm disclosed in Smith & Waterman, 1981, by the homology alignment algorithm disclosed in Needleman & Wunsch, 1970, by the search for similarity method disclosed in Pearson & Lipman, 1988, by computerized implementations of these

30      algorithms (GAP, BESTFIT, FASTA, and TFASTA in the GCG® WISCONSIN PACKAGE™, available from Accelrys Inc., San Diego, California, United States of America), or by visual inspection. *See* generally, Ausubel, 1995.

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A representative algorithm for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul et al., 1990. Software for performing BLAST analyses is publicly available through the website of the National Center for Biotechnology

5      Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold. These initial

10     neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues;

15     always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when the cumulative alignment score falls off by the quantity X from its maximum achieved value, the cumulative score goes to zero or below due to the

20     accumulation of one or more negative-scoring residue alignments, or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) = 11, an expectation (E) = 10, a cutoff of 100, M = 5, N = -4, and a comparison of

25     both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix. See Henikoff & Henikoff, 1992.

In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two

30     sequences. See e.g., Karlin & Altschul, 1993. One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a

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test nucleic acid sequence is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid sequence to the reference nucleic acid sequence is less than about 0.1 in some embodiments, less than about 0.01 in some embodiments, and less than about 0.001 in some embodiments.

5

II.D.    Peptide Ligands

A peptide of the presently disclosed subject matter can be subject to various changes, substitutions, insertions, and deletions where such changes provide for certain advantages in its use. Thus, the term "peptide"

10 encompasses any of a variety of forms of peptide derivatives, which include amides, conjugates with proteins, cyclized peptides, polymerized peptides, conservatively substituted variants, analogs, fragments, peptoids, chemically modified peptides, and peptide mimetics. The term "peptide ligand" refers to a peptide as defined hereinabove that binds to an EGFR heterodimer or

15 prevents the dimerization of EGFR and another ERBB family member. The modifications disclosed herein can also be applied as desired and as appropriate to antibodies, including scFv antibodies.

Peptides of the presently disclosed subject matter can comprise naturally occurring amino acids, synthetic amino acids, genetically encoded

20 amino acids, non-genetically encoded amino acids, and combinations thereof. Peptides can include both L-form and D-form amino acids.

Representative non-genetically encoded amino acids include but are not limited to 2-aminoadipic acid; 3-aminoadipic acid; β-aminopropionic acid; 2-aminobutyric acid; 4-aminobutyric acid (piperidinic acid); 6-aminocaproic

25 acid; 2-aminoheptanoic acid; 2-aminoisobutyric acid; 3-aminoisobutyric acid; 2-aminopimelic acid; 2,4-diaminobutyric acid; desmosine; 2,2'-diaminopimelic acid; 2,3-diaminopropionic acid; N-ethylglycine; N-ethylasparagine; hydroxylysine; allo-hydroxylysine; 3-hydroxyproline; 4-hydroxyproline; isodesmosine; allo-isoleucine; N-methylglycine (sarcosine);

30 N-methylisoleucine; N-methylvaline; norvaline; norleucine; and ornithine.

Representative derivatized amino acids include for example, those molecules in which free amino groups have been derivatized to form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-

butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups can be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups can be derivatized to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine can

5     be derivatized to form N-im-benzylhistidine.

The term "conservatively substituted variant" refers to a peptide comprising an amino acid residue sequence substantially identical to a sequence of a reference ligand of an EGFR heterodimer in which one or more residues have been conservatively substituted with a functionally

10    similar residue and which displays the EGFR heterodimer binding activity or the ability to prevent formation of an EGFR heterodimer as described herein. The phrase "conservatively substituted variant" also includes peptides wherein a residue is replaced with a chemically derivatized residue, provided that the resulting peptide displays activities disclosed herein.

15    Examples of conservative substitutions include the substitution of one non-polar (hydrophobic) residue such as isoleucine, valine, leucine or methionine for another; the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, between glycine and serine; the substitution of one basic

20    residue such as lysine, arginine or histidine for another; or the substitution of one acidic residue, such as aspartic acid or glutamic acid for another.

Peptides of the presently disclosed subject matter also include peptides comprising one or more additions and/or deletions or residues relative to the sequence of a peptide whose sequence is disclosed herein,

25    so long as the requisite activities of the peptide are maintained. The term "fragment" refers to a peptide comprising an amino acid residue sequence shorter than that of a peptide disclosed herein.

Additional residues can also be added at either terminus of a peptide for the purpose of providing a "linker" by which the peptides of the presently

30    disclosed subject matter can be conveniently affixed to a label or solid matrix, or carrier. Amino acid residue linkers are usually at least one residue and can be 40 or more residues, more often 1 to 10 residues, but do alone not constitute receptor tyrosine kinase ligands. Typical amino acid residues

used for linking are tyrosine, cysteine, lysine, glutamic and aspartic acid, or the like.  In addition, a peptide can be modified by terminal-$NH_2$ acylation (e.g., acetylation, or thioglycolic acid amidation) or by terminal-carboxylamidation (e.g., with ammonia, methylamine, and the like terminal

5    modifications).  Terminal modifications are useful, as is well known, to reduce susceptibility by proteinase digestion, and therefore serve to prolong half-life of the peptides in solutions, particularly biological fluids where proteases can be present.

Peptide cyclization is also a useful terminal modification because of

10    the stable structures formed by cyclization and in view of the biological activities observed for such cyclic peptides as described herein.  An exemplary method for cyclizing peptides is described by Schneider & Eberle, 1993.  Typically, tertbutoxycarbonyl protected peptide methyl ester is dissolved in methanol and sodium hydroxide solution are added and the

15    admixture is reacted at 20°C to hydrolytically remove the methyl ester protecting group.  After evaporating the solvent, the tertbutoxycarbonyl protected peptide is extracted with ethyl acetate from acidified aqueous solvent.  The tertbutoxycarbonyl protecting group is then removed under mildly acidic conditions in dioxane cosolvent. The unprotected linear peptide

20    with free amino and carboxyl termini so obtained is converted to its corresponding cyclic peptide by reacting a dilute solution of the linear peptide, in a mixture of dichloromethane and dimethylformamide, with dicyclohexylcarbodiimide in the presence of 1-hydroxybenzotriazole and N-methylmorpholine.  The resultant cyclic peptide is then purified by

25    chromatography.

The term "peptoid" as used herein refers to a peptide wherein one or more of the peptide bonds are replaced by pseudopeptide bonds including but not limited to a carba bond ($CH_2$-$CH_2$), a depsi bond (CO-O), a hydroxyethylene bond (CHOH-$CH_2$), a ketomethylene bond (CO-$CH_2$), a

30    methylene-oxy bond ($CH_2$-O), a reduced bond ($CH_2$-NH), a thiomethylene bond ($CH_2$-S), a thiopeptide bond (CS-NH), and an N-modified bond (-NRCO-). See e.g. Corringer et al., 1993; Garbay-Jaureguiberry et al., 1992; Tung et al., 1992; Urge et al., 1992; Pavone et al., 1993.

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Peptides of the presently disclosed subject matter, including peptoids, can be synthesized by any of the techniques that are known to those skilled in the art of peptide synthesis. Synthetic chemistry techniques, such as a solid-phase Merrifield-type synthesis, can be used for reasons of purity,

5    antigenic specificity, freedom from undesired side products, ease of production, and the like. A summary of representative techniques can be found in Stewart & Young, 1969; Merrifield, 1969; Fields & Noble, 1990; and Bodanszky, 1993. Solid phase synthesis techniques can be found in Andersson et al., 2000, references cited therein, and in U.S. Patent Nos.

10   6,015,561; 6,015,881; 6,031,071; and 4,244,946. Peptide synthesis in solution is described by Schröder & Lübke, 1965. Appropriate protective groups usable in such synthesis are described in the above texts and in McOmie, 1973. Peptides that include naturally occurring amino acids can also be produced using recombinant DNA technology. In addition, peptides

15   comprising a specified amino acid sequence can be purchased from commercial sources (e.g., Biopeptide Co., LLC of San Diego, California, United States of America and PeptidoGenics of Livermore, California, United States of America).

The term "peptide mimetic" as used herein refers to a ligand that

20   mimics the biological activity of a reference peptide, by substantially duplicating the activities of the reference peptide, but it is not a peptide or peptoid. In some embodiments, a peptide mimetic has a molecular weight of less than about 700 daltons.

A peptide mimetic can be designed by: (a) identifying the

25   pharmacophoric groups responsible for the activities of a' peptide; (b) determining the spatial arrangements of the pharmacophoric groups in the active conformation of the peptide; and (c) selecting a pharmaceutically acceptable template upon which to mount the pharmacophoric groups in a manner that allows them to retain their spatial arrangement in the active

30   conformation of the peptide. For identification of pharmacophoric groups responsible for an activity, mutant variants of the peptide can be prepared and assayed for the activity. Alternatively or in addition, the three-dimensional structure of a complex of the peptide and its target molecule can

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be examined for evidence of interactions, for example the fit of a peptide side chain into a cleft of the target molecule, potential sites for hydrogen bonding, etc. The spatial arrangements of the pharmacophoric groups can be determined by NMR spectroscopy or X-ray diffraction studies. An initial

5      three-dimensional model can be refined by energy minimization and molecular dynamics simulation. A template for modeling can be selected by reference to a template database and will typically allow the mounting of 2-8 pharmacophores. A peptide mimetic is identified wherein addition of the pharmacophoric groups to the template maintains their spatial arrangement

10     as in the peptide.

A peptide mimetic can also be identified by assigning a hashed bitmap structural fingerprint to the peptide based on its chemical structure, and determining the similarity of that fingerprint to that of each compound in a broad chemical database. The fingerprints can be determined using

15     fingerprinting software commercially distributed for that purpose by Daylight Chemical Information Systems, Inc. (Mission Viejo, California, United States of America) according to the vendor's instructions. Representative databases include but are not limited to SPREI'95 (InfoChem GmbH of München, Germany), Index Chemicus (ISI of Philadelphia, Pennsylvania,

20     United States of America), World Drug Index (Derwent of London, United Kingdom), TSCA93 (United States Envrionmental Protection Agency), MedChem (Biobyte of Claremont, California, United States of America), Maybridge Organic Chemical Catalog (Maybridge of Cornwall, England), Available Chemicals Directory (MDL Information Systems of San Leandro,

25     California, United States of America), NCI96 (United States National Cancer Institute), Asinex Catalog of Organic Compounds (Asinex Ltd. of Moscow, Russia), and NP (InterBioScreen Ltd. of Moscow, Russia). A peptide mimetic of a reference peptide is selected as comprising a fingerprint with a similarity (Tanamoto coefficient) of at least 0.85 relative to the fingerprint of

30     the reference peptide. Such peptide mimetics can be tested for bonding to an irradiated tumor using the methods disclosed herein. Additional techniques for the design and preparation of peptide mimetics can be found

in U.S. Patent Nos. 5,811,392; 5,811,512; 5,578,629; 5,817,879; 5,817,757; and 5,811,515.

Any peptide or peptide mimetic of the presently disclosed subject matter can be used in the form of a pharmaceutically acceptable salt.
5   Suitable acids which are capable of the peptides with the peptides of the presently disclosed subject matter include inorganic acids such as trifluoroacetic acid (TFA), hydrochloric acid (HCl), hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, phosphoric acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid,
10   malonic acid, succinic acid, maleic acid, fumaric acid, anthranilic acid, cinnamic acid, naphthalene sulfonic acid, sulfanilic acid or the like.

Suitable bases capable of forming salts with the peptides of the presently disclosed subject matter include inorganic bases such as sodium hydroxide, ammonium hydroxide, potassium hydroxide and the like; and
15   organic bases such as mono-di- and tri-alkyl and aryl amines (e.g. triethylamine, diisopropyl amine, methyl amine, dimethyl amine and the like), and optionally substituted ethanolamines (e.g. ethanolamine, diethanolamine and the like).

III.   Applications

20   The presently disclosed subject matter also provides a method for suppressing the growth of a tumor associated with EGFR heterodimer activity in a subject, the method comprising administering to the subject bearing the tumor associated with EGFR heterodimer activity an effective amount of a EGFR heterodimer binding compound as disclosed herein,
25   whereby growth of the tumor is suppressed

In some embodiments, the method comprises contacting a tumor cell in a tumor with an EGFR heterodimer binding compound (for example, an antibody, peptide, or small molecule that specifically binds to an EGFR/ERBB heterodimer) under conditions sufficient to allow the EGFR
30   heterodimer binding compound to inhibit the activity of the EGFR heterodimer. In some embodiments, the EGFR heterodimer binding compound is administered directly to a subject having a tumor. In some embodiments, a vector (for example, an expression vector, an adenoviral

vector, or a retroviral vector) encoding an EGFR heterodimer binding compound is administered to the subject. For example, EGFR heterodimer binding compounds can be useful in the treatment of both primary and metastatic solid tumors and carcinomas of the breast; colon; rectum; lung;

5   oropharynx; hypopharynx; esophagus; stomach; pancreas; liver; gallbladder; bile ducts; small intestine; urinary tract including kidney, bladder and urothelium; female genital tract including cervix, uterus, ovaries, choriocarcinoma and gestational trophoblastic disease; male genital tract including prostate, seminal vesicles, testes and germ cell tumors; endocrine

10  glands including thyroid, adrenal, and pituitary; skin including hemangiomas, melanomas, sarcomas arising from bone or soft tissues and Kaposi's sarcoma; tumors of the brain, nerves, eyes, and meninges including astrocytomas, gliomas, glioblastomas, retinoblastomas, neuromas, neuroblastomas, Schwannomas and meningiomas; solid tumors arising from

15  hematopoietic malignancies such as leukemias and including chloromas, plasmacytomas, plaques and tumors of mycosis fungoides and cutaneous T-cell lymphoma/leukemia; lymphomas including both Hodgkin's and non-Hodgkin's lymphomas.

III.A.  Subjects

20      . The subject treated in the presently disclosed subject matter in its many embodiments is desirably a human subject, although it is to be understood that the principles of the presently disclosed subject matter indicate that the presently disclosed subject matter is effective with respect to invertebrate and to all vertebrate species, including mammals, which are

25  intended to be included in the term "subject". Moreover, a mammal is understood to include any mammalian species in which treatment or prevention of cancer is desirable, particularly agricultural and domestic mammalian species.

The methods of the presently disclosed subject matter are particularly

30  useful in the treatment of warm-blooded vertebrates. Thus, the presently disclosed subject matter concerns mammals and birds.

More particularly provided is the treatment of mammals such as humans, as well as those mammals of importance due to being endangered

(such as Siberian tigers), of economic importance (animals raised on farms for consumption by humans) and/or social importance (animals kept as pets or in zoos) to humans, for instance, carnivores other than humans (such as cats and dogs), swine (pigs, hogs, and wild boars), ruminants (such as

5     cattle, oxen, sheep, giraffes, deer, goats, bison, and camels), and horses. Also provided is the treatment of birds, including the treatment of those kinds of birds that are endangered, kept in zoos, as well as fowl, and more particularly domesticated fowl, i.e., poultry, such as turkeys, chickens, ducks, geese, guinea fowl, and the like, as they are also of economic importance to

10    humans. Thus, contemplated is the treatment of livestock, including, but not limited to, domesticated swine (pigs and hogs), ruminants, horses, poultry, and the like.

III.B.   Formulation

The EGFR heterodimer binding compounds of the presently disclosed

15    subject matter, or vectors encoding the same, comprise in some embodiments a composition that includes a pharmaceutically acceptable carrier.

For example, suitable formulations can include aqueous and non-aqueous sterile injection solutions which can contain anti-oxidants, buffers,

20    bacteriostats, bactericidal antibiotics and solutes which render the formulation isotonic with the bodily fluids of the intended recipient; and aqueous and non-aqueous sterile suspensions which can include suspending agents and thickening agents. The formulations can be presented in unit-dose or multi-dose containers, for example sealed

25    ampoules and vials, and can be stored in a frozen or freeze-dried (lyophilized) condition requiring only the addition of sterile liquid carrier, for example water for injections, immediately prior to use. Some exemplary ingredients are SDS, in some embodiments in the range of 0.1 to 10 mg/ml, in some embodiments about 2.0 mg/ml; and/or mannitol or another sugar, for

30    example in the range of 10 to 100 mg/ml, in some embodiments about 30 mg/ml; and/or phosphate-buffered saline (PBS).

It should be understood that in addition to the ingredients particularly mentioned above the formulations of this presently disclosed subject matter

can include other agents conventional in the art having regard to the type of formulation in question. For example, sterile pyrogen-free aqueous and non-aqueous solutions can be used.

The therapeutic regimens and compositions of the presently disclosed subject matter can be used with additional adjuvants or biological response modifiers including, but not limited to, the cytokines IFN-α, IFN-γ, IL2, IL4, IL6, TNF, or other cytokine affecting immune cells. In accordance with this aspect of the presently disclosed subject matter, the disclosed EGFR heterodimer binding compounds or vectors encoding the same can be administered in combination therapy with one or more of these cytokines.

III.C.  Administration

Administration of the compositions of the presently disclosed subject matter can be by any method known to one of ordinary skill in the art, including, but not limited to intravenous administration, intrasynovial administration, transdermal administration, intramuscular administration, subcutaneous administration, topical administration, rectal administration, intravaginal administration, intratumoral administration, oral administration, buccal administration, nasal administration, parenteral administration, inhalation, and insufflation. In some embodiments, suitable methods for administration of a nucleic acid molecule of the presently disclosed subject matter (for example, using an expression vector) include but are not limited to intravenous or intratumoral injection. Alternatively, a nucleic acid molecule can be deposited at a site in need of treatment in any other manner, for example by spraying a composition comprising a nucleic acid molecule within the pulmonary pathways. The particular mode of administering a composition of the presently disclosed subject matter depends on various factors, including the distribution and abundance of cells to be treated, the vector employed, additional tissue- or cell-targeting features of the vector, and mechanisms for metabolism or removal of the vector from its site of administration. For example, relatively superficial tumors can be injected intratumorally. By contrast, internal tumors can be treated by intravenous injection.

In some embodiments, the method of administration encompasses features for regionalized delivery or accumulation at the site in need of treatment. In some embodiments, an EGFR heterodimer binding compound or expression vector encoding the same is delivered intratumorally. In some

5   embodiments, selective delivery of an EGFR heterodimer binding compound or vector encoding the same to a tumor is accomplished by intravenous injection of the construct

For delivery of an EGFR heterodimer binding compound or vector encoding the same to pulmonary pathways, an EGFR heterodimer binding

10   compound or vector encoding the same of the presently disclosed subject matter can be formulated as an aerosol or coarse spray. Methods for preparation and administration of aerosol or spray formulations can be found, for example, in Cipolla *et al.*, 2000, and in U.S. Patent Nos. 5,858,784; 6,013,638; 6,022,737; and 6,136,295.

15   III.D.   Dose

An effective dose of a composition of the presently disclosed subject matter is administered to a subject in need thereof. A "therapeutically effective amount" is an amount of the composition sufficient to produce a measurable response (*e.g.*, a decrease in the growth rate of a tumor). In

20   some embodiments, an activity that inhibits tumor growth is measured. Actual dosage levels of active ingredients in the compositions of the presently disclosed subject matter can be varied so as to administer an amount of the active compound(s) that is effective to achieve the desired therapeutic response for a particular subject. The selected dosage level will

25   depend upon the activity of the therapeutic composition, the route of administration, combination with other drugs or treatments, the severity of the condition being treated, and the condition and prior medical history of the subject being treated. However, it is within the skill of the art to start doses of the compound at levels lower than required to achieve the desired

30   therapeutic effect and to gradually increase the dosage until the desired effect is achieved.

The potency of a composition can vary, and therefore a "therapeutically effective" amount can vary. However, using the assay

methods described herein below, one skilled in the art can readily assess the potency and efficacy of a candidate modulator of this presently disclosed subject matter and adjust the therapeutic regimen accordingly.

After review of the disclosure of the presently disclosed subject matter presented herein, one of ordinary skill in the art can tailor the dosages to an individual patient, taking into account the particular formulation, method of administration to be used with the composition, and tumor size. Further calculations of dose can consider patient height and weight, severity and stage of symptoms, and the presence of additional deleterious physical conditions. Such adjustments or variations, as well as evaluation of when and how to make such adjustments or variations, are well known to those of ordinary skill in the art of medicine.

For example, for local administration of viral vectors, previous clinical studies have demonstrated that up to $10^{13}$ plaque-forming units (pfu) of virus can be injected with minimal toxicity. In human patients, $1 \times 10^9 - 1 \times 10^{13}$ pfu are routinely used (see Habib et al., 1999). To determine an appropriate dose within this range, preliminary treatments can begin with $1 \times 10^9$ pfu, and the dose level can be escalated in the absence of dose-limiting toxicity. Toxicity can be assessed using criteria set forth by the National Cancer Institute and is reasonably defined as any grade 4 toxicity or any grade 3 toxicity persisting more than 1 week. Dose is also modified to maximize anti-tumor or anti-angiogenic activity. Representative criteria and methods for assessing anti-tumor and/or anti-angiogenic activity are described herein below. With replicative virus vectors, a dosage of about $1 \times 10^7$ to $1 \times 10^8$ pfu can be used in some instances.

An expression construct as disclosed herein can be packaged into a vector (for example, an viral vector including, but not limited to an adenovirus vector, an adeno-associated virus vector, or a retroviral vector) and the prepared virus titer reaches at least $1 \times 10^6 - 1 \times 10^7$ pfu/ml. In some embodiments, the viral vector is administered in the amount of 1.0 pfu/target cell. Thus, administration of a minimal level of viral vector to thereby provide a therapeutic level of an EGFR heterodimer binding compound encoded by a viral vector comprises an aspect of the presently disclosed subject matter.

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## Examples

The following Examples have been included to illustrate modes of the presently disclosed subject matter. Certain aspects of the following Examples are described in terms of techniques and procedures found or

5   contemplated by the present co-inventors to work well in the practice of the presently disclosed subject matter. These Examples illustrate standard laboratory practices of the co-inventors. In light of the present disclosure and the general level of skill in the art, those of skill will appreciate that the following Examples are intended to be exemplary only and that numerous

10  changes, modifications, and alterations can be employed without departing from the scope of the presently disclosed subject matter.

## Example 1

### $Egfr^{Wa5}$ Mutation Identification

15  All exons (Reiter *et al.*, 2001), along with immediate flanking regions, of *Egfr* were amplified by polymerase chain reaction (PCR) from $Egfr^{Wa5}$/+, BALB/c, C3H, and C57BL/6 genomic DNA using intronic primers that were also used for subsequent sequence analysis. PCR products were purified using the multiscreen PCR 96-well filtration system (Millipore Corp., Billerica,

20  Massachusetts, United States of America) on a BIOMEK® 2000 robotic platform (Beckman Coulter, Inc., Fullerton, California, United States of America) and sequenced directly using BIG DYE™ terminator cycle sequencing (Applied Biosystems, Inc., Foster City, California, United States of America). Sequences were analyzed using the SEQUENCER™ program

25  (Gene Codes Corp., Ann Arbor, Michigan, United States of America) to identify the causative mutation in $Egfr^{Wa5}$.

## Example 2

### Mouse Strains, Crosses, and Genotyping

30  The $Egfr^{Wa5}$ mutation was maintained by intercrossing $Egfr^{Wa5}$/+ mice on a mixed genetic background containing contributions from BALB/c, C3H, and C57BL/6J. The $Egfr^{wa2}$/+ and $Egfr^{tm1Mag}$/+ (Threadgill *et al.*, 1995) mutations were maintained on C57BL/6J congenic or 129S6/SvEvTAC

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isogenic backgrounds, respectively. *Tgfa*$^{tm1Dcl}$ null mice were maintained on a mixed genetic background of 129S6/SvEvTAC and C57BL/6J (Luetteke *et al.*, 1993). The *Apc*$^{Min}$ mutation (Moser *et al.*, 1993) was maintained as congenic on a C57BL/6J background.

Complementation studies were performed by crossing *Egfr*$^{tm1Mag}$/+ females with *Egfr*$^{Wa5}$/+ males. *Egfr*$^{Wa5}$/+ female mice were bred to *Apc*$^{Min}$/+ male mice, producing *Apc*$^{Min}$/+ mice on wild type and *Egfr*$^{Wa5}$/+ backgrounds. To generate *Tgfa*$^{tm1Dcl/tm1Dcl}$, *Egfr*$^{Wa5}$/+ double-mutant mice, Tgfa$^{tm1Dcl/tm1Dcl}$ females were mated to *Tgfa*$^{tm1Dcl}$/+, *Egfr*$^{Wa5}$/+ males. In order to generate compound heterozygous animals, *Egfr*$^{wa2}$/+ females were crossed to *Egfr*$^{Wa5}$/+ males. The morning a vaginal plug was detected was defined as 0.5 day of embryonic development (0.5 dpc). Mice were fed Purina Mills Lab Diet 5058 (Purina Mills, LLC, St. Louis, Missouri, United States of America) under specific pathogen-free conditions in an American Association for the Accreditation of Lab Animal Care approved facility. Mice were euthanized by $CO_2$ asphyxiation.

Embryonic yolk sacs from embryos or tail clips from neonates were used to extract DNA for genotype determination by PCR. The genotype for the *Egfr*$^{Wa5}$ allele was determined by PCR using three primers: mEgfr-Ex21-S5, 5'-gcatgtcaagatcacaga-3' (SEQ ID NO: 7); mEgfr-Ex21-S6, 5'-gcatgtcaagatcacagg-3' (SEQ ID NO: 8); and mEgfr-In21-As1, 5'-tagagaatgaccctgacgag-3' (SEQ ID NO: 9). The mEgfr-Ex21-S5 and mEgfr-In21-As1 primers amplify a 228-base pair (bp) product specific for the wild type *Egfr* allele and primers mEgfr-Ex21-S6 and mEgfr-In21-As1 amplify a 228-bp product specific for the *Egfr*$^{Wa5}$ allele. Genotypes for the *Egfr*$^{tm1Mag}$, *Egfr*$^{wa2}$, *Apc*$^{Min}$, *Mom1*, and *Tgfa*$^{tm1Dcl}$ alleles were determined by PCR as previously described (Luetteke *et al.*, 1993; Roberts *et al.*, 2001).

## Example 3

### *In vivo* Phosphorylation Assays

Neonatal pups were injected subcutaneously with 10 μl/g body weight of phosphate-buffered saline (PBS) or 0.5, 1.0, or 10 μg/g body weight of EGF (R&D Systems Inc., Minneapolis, Minnesota, United States of America)

in PBS. After 10 minutes, liver and skin were harvested, frozen in liquid nitrogen, and stored at -80°C. The frozen tissues were homogenized in 5-10 volumes (5-10 ml/g tissue) of homogenization buffer consisting of 20 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM PMSF,

5    10 µg/ml of leupeptin, 10 µg/ml of aprotinin, 1 mM sodium vanadate, and 10 mM $\beta$-glycerophosphate at 4°C. The tissue lysates were cleared by centrifugation for 10 minutes at 4°C and protein concentrations were determined by the Bradford assay (Bio-Rad Laboratories, Hercules, California, United States of America). An equal amount of protein lysate (15

10   µg) was separated by denaturing 7.5% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (Bio-Rad Laboratories). Protein blots were incubated with recombinant antiphosphotyrosine antibody conjugated with horseradish peroxidase (BD Biosciences Pharmingen, San Diego, California, United

15   States of America) or polyclonal rabbit anti-EGFR antibody (Lab Vision/NeoMarkers, Montréal, Québec, Canada) and detected with an enhanced chemiluminesence system (Amersham Biosciences, Piscataway, New Jersey, United States of America).

20
## Example 4
### Cell Culture and Transfection

Chinese hamster ovary (CHO) cells were grown in Dulbecco's modified Eagle's 1 medium (DMEM) supplemented with 10% fetal bovine serum (FBS), nonessential amino acids, and penicillin/streptomycin. CHO

25   cells in 60-mm dishes at 50%–70% confluency were transfected with human EGFR expression vectors in pcDNA3.1 (Invitrogen Corporation, Carlsbad, California, United States of America) using FuGENE 6 transfection reagent (Roche Applied Science, Palo Alto, California, United States of America) and cell extracts were prepared at 24 hour after transfection as described

30   previously (Fitch et al., 2003). The Asp855Gly (Wa5) change was introduced into a human EGFR expression vector by site-directed mutagenesis using GENEEDITOR™ (Promega Corp., Madison, Wisconsin, United States of America) and the entire coding regions were verified by

sequence analysis. An equal amount of protein (12 μg) was separated by denaturing 6% SDS-PAGE and analyzed by western blot analysis as described above.

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<div align="center">

## Example 5

### Chemical Cross-linking

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Chemical cross-linking of EGFR was carried out as described previously (Qian et al., 1994). Briefly, CHO cells transfected with EGFR expression vectors in 6-well plates for 24 hours were washed twice with cold

10   PBS. One milliliter of PBS containing 2 mM bis(sulfosuccinimidyl) suberate (BS₃; Pierce Biotechnology, Inc., Rockford, Illinois, United States of America) was added and incubated at room temperature for 30 minutes with rocking of the plates. The reaction was stopped by washing twice with cold 20 mM Tris-HCl, pH 7.4/150 mM NaCl and cell lysates were prepared as described

15   above. Protein concentration was measured using the Bradford assay (Bio-Rad Laboratories, Hercules, California, United States of America). An equal amount (12 μg) of protein was separated by 6% SDS-PAGE, transferred to PVDF membranes and subjected to western blot analysis as described above.

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## Example 6

### FLAG-tag and Immunoprecipitation-Western Blot

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A *Xba* I to *Dra* III fragment of the human EGFR expression vector was replaced with a *Sac* I to *Dra* III fragment of pBK-Flag-EGFR (Kim et al.,

25   2003) containing the FLAG epitope immediately after the signal sequence to generate N-terminal FLAG-tagged wild-type (fWt), kd (fkd) and Wa5 (fWa5) EGFR expression vectors. Wild-type *EGFR* expression vectors were co-transfected with an equal quantity (0.2 μg) of $EGFR^f$, $EGFR^{fkd}$, or $EGFR^{fWa5}$ expression vector and cell extracts were prepared 24 hours after transfection

30   as described above. Cell lysates (200 μg) were immunoprecipitated with 5 μg of monoclonal anti-FLAG M2 antibody (Sigma-Aldrich Co., St. Louis, Missouri, United States of America) overnight at 4°C and then for 2 hours after addition of 30 μl of Protein G-agarose (Pierce Biotechnology, Inc.).

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Immunoprecipitated complexes were washed three times with TBS buffer (50 mM Tris-HCl, pH 7.4/150 mM NaCl) and the bound proteins were eluted by boiling for 5 minutes in 2x SDS sample buffer (125 mM Tris-HCl, pH 6.8/4% SDS/20% glycerol/0.05% bromophenol blue). For the immunoprecipitation of EGFR or ERBB2, cell lysates (100 µg) were incubated overnight with 5 µg of monoclonal anti-EGFR antibody (clone 528) or 3 µg of polyclonal anti-ERBB2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, California, United States of America) at 4°C. After 2 hours of incubation with 30 µl of Protein G-agarose, immunoprecipitated complexes were washed four times with HNTG buffer (20 mM HEPES, pH 7.5/150 mM NaCl/0.1% Triton X-100/10% glycerol) and the bound proteins were eluted as described above. One-sixth of the immunoprecipitate volumes were separated by 6% SDS-PAGE, transferred to PVDF membranes and subjected to western blot analysis. For the detection of the FLAG epitope, the same antibody was used for western blot analysis as for immunoprecipitation.

## Example 7

### Intestinal Macroadenoma Analysis

The small intestines from pylorus to cecum and the colons were dissected from 3-month-old mice, flushed gently with PBS to remove fecal material, cut longitudinally, and splayed flat on Whatman 3MM paper (Whatman Inc., Florham Park, New Jersey, United States of America). The gastrointestinal tracts were fixed overnight at 4°C in 10% neutral buffered formalin (Sigma-Aldrich Co., St. Louis, Missouri, United States of America) before storing in 70% ethanol. The number and diameter of macroadenoma polyps were characterized as previously described (Roberts et al., 2001). The Mann–Whitney paired rank sum test was used to analyze the association between genotype and polyp number and size. Statistical analysis was performed using StatView (SAS Institute Inc., Cary, North Carolina, United States of America).

<u>Discussion of Examples 1-7</u>

   <u>Wa5 has a point mutation in a highly conserved residue within *Egfr*</u>
<u>and causes embryonic and perinatal lethality.</u>  Previous analysis of ENU-
induced mutations causing dominant visible phenotypes detected *Wa5* as a

5  mutation that causes open eyes at birth, curly whiskers, and a wavy coat
similar to the recessive *Tgfa*[wa1] and *Egfr*[wa2] mutations (Thaung *et al.*, 2002).
Since genetic mapping localized the mutation to proximal Chromosome 11
near the *Egfr* locus, a complementation cross was performed with an
*Egfr*[tm1Mag] null allele (*see* Table 1).  The *Wa5* allele failed to complement

10  *Egfr*[tm1Mag], supporting the fact that *Wa5* is a new allele of *Egfr* (*Egfr*[Wa5]).  The
entire coding region from EgfrWa5 transcripts was sequenced, and a single-
point mutation was identified that creates a missense mutation (GAT to
GGT) in exon 21; this change results in a D833G amino acid change within
the tyrosine kinase domain (*see* Figure 1A), altering a DFG motif that is

15  ubiquitously conserved in all functional protein kinases (Hanks *et al.*, 1988).
The Lys723 residue binds Mn•ATP that is aligned within the active site by
Asp833 for catalysis at Asp815 (Coker *et al.*, 1994).  The previously
characterized *Egfr*[wa2] (Helmrath *et al.*, 1997; Egger *et al.*, 2000; Wang *et al.*,
2003) and *Egfr*[Dsk5] (Fitch *et al.*, 2003) mutations, which result in changes at

20  Val743 and Leu839 probably alter the structure of the active site since they
are in an alpha-helix (ac) and the activation loop, respectively, rather than
the catalytic loop (*see* Figure 1B).

<div align="center">Table 1</div>

<u>Wa5 and Egfr[tm1Mag] Complementation Cross – Results from 3 Litters</u>

| Genotypes | | | |
|---|---|---|---|
| +/+ | Wa5/+ | Egfrtm1 Mag/+ | Egfrtm1Mag/Wa5 |
| 7 | 14 | 6 | 0 |

25   Tyrosine kinases lacking the conserved DFG motif are kinase dead
(Kroiher *et al.*, 2001) and hypothesized to negatively regulate active kinases
when oligomerized.  In this manner, *Egfr*[Wa5] appears to be acting as an
antimorphic allele, distinctly different than the previously characterized
dominant hypermorphic mutation *Egfr*[Dsk5] (Fitch *et al.*, 2003).

Since $Egfr^{Wa5}$ failed to complement the $Egfr^{tm1Mag}$ null allele, the survival of $Egfr^{Wa5}$ homozygous embryos generated from $Egfr^{Wa5}/+$ intercrosses was analyzed. Morphologically normal $Egfr^{Wa5/Wa5}$ embryos were observed at 10.5 dpc in expected Mendelian ratios (*see* Table 2).

5 Lethality of the majority of $Egfr^{Wa5/Wa5}$ embryos (71%) occurs between 10.5 and 12.5 dpc; a similar age of embryonic lethality was found to be caused by homozygosity for the $Egfr^{tm1Mag}$ null allele (Threadgill *et al.*, 1995). Morphologically abnormal and severely degenerated embryos at 10.5-14.5 dpc were either $Egfr^{Wa5}/+$ (25%) or $Egfr^{Wa5/Wa5}$ (75%). Although a few

10 $Egfr^{Wa5/Wa5}$ embryos developed to later stages of gestation, no $Egfr^{Wa5/Wa5}$ embryos survived postnatally; despite being grossly normal in size, three newborn $Egfr^{Wa5/Wa5}$ pups were found dead shortly after birth. This result is consistent with that observed for the $Egfr^{tm1Mag}$ null allele where embryos homozygous for $Egfr^{tm1Mag}$ display variable embryonic and early postnatal

15 lethality that is dependent upon genetic background (Strunk *et al.*, 2004).

### Table 2

Viability of $Egfr^{Wa5}$ Homozygotes from $Egfr^{Wa5}/+$ Intercrosses

| Stage[a] | Genotypes[b] | | | Litters |
|---|---|---|---|---|
| | +/+ | $Egfr^{Wa5}/+$ | $Egfr^{Wa5/Wa5}$ | |
| 10.5 dpc | 12 (0) | 26 (2) | 14 (4) | 8 |
| 12.5 dpc | 22 (0) | 40 (0) | 6 (3) | 10 |
| 14.5 dpc | 13 (0) | 30 (1) | 6 (2) | 8 |
| P0 | 20 | 40 | 3 | 11 |
| P21 | 20 | 39 | 0 | 11 |

[a] dpc, embryonic day and P, postnatal day.

[b] Number of normal (abnormal) embryos or live pups.

20 $Egfr^{Wa5}$ allele enhances the recessive $Egfr^{wa2}$ hypomorphic and $Tgfa^{TM1Dcl}$ null phenotypes. On a mixed genetic background, $Egfr^{wa2}$ homozygous pups surviving to adulthood are fertile, and show defects only in hair follicle morphogenesis (Luetteke *et al.*, 1994). Therefore, to determine the effects of further reduction in EGFR activity, $Egfr^{wa2}/+$ females

25 were crossed with $Egfr^{Wa5}/+$ males to generate $Egfr^{wa2/Wa5}$ compound heterozygotes (*see* Table 3). When compared to wild type littermates, only

half of the predicted $Egfr^{wa2/Wa5}$ compound pups were born, although those surviving were normal in size at birth. Nine of 16 $Egfr^{wa2/Wa5}$ pups died during the first two days after birth, while three $Egfr^{wa2/Wa5}$ pups became progressively more runted, dying between two and three weeks of age. Four
5   of 16 $Egfr^{wa2/Wa5}$ compound mutants, including three males and one female, survived as long as three months after birth. These $Egfr^{wa2/Wa5}$ compound mutants showed severe growth retardation with a body weight ranging from 30% to 50% of the control littermates. Although hair development of $Egfr^{wa2/Wa5}$ pups was initially similar to that of $Egfr^{wa2/wa2}$ pups, $Egfr^{wa2/Wa5}$
10  compound mutant mice underwent a complete hair loss around 8 weeks of age; new hair growth resumed at 2 months of age. Craniofacial malformations, including an underdeveloped lower jaw and narrow, elongated, and asymmetric snouts, were also observed in $Egfr^{wa2/Wa5}$ compound-mutant mice, similar to the phenotype described previously in
15  $Egfr$ nullizygous mice (Miettinen et al., 1999). The only female $Egfr^{wa2/Wa5}$ mutant that survived postweaning showed a delay in vaginal opening until 11 weeks of age, an increase in severity of a phenotype reported for $Egfr^{wa2/wa2}$ mice that show delayed vaginal opening at 7.5 weeks (Apostolakis et al., 2000). Neither gender produced offspring by 3 months of age, suggesting
20  they might also have fertility defects.

Similar to the $Egfr^{wa2}$ mutation, transforming growth factor α (TGFA) deficiency gives rise to coat and eye abnormalities (Luetteke et al., 1993; Mann et al., 1993). Since TGFA is one of the major ligands for EGFR, the phenotypic severity of $Tgfa^{tm1Dcl/tm1Dcl}$, $Egfr^{Wa5}/+$ double mutants produced by
25  crossing $Tgfa^{tm1Dcl}$ nullizygous females to $Egfr^{Wa5}/+$, $Tgfa^{tm1Dcl}/+$ males. Double-mutant $Tgfa^{tm1Dcl/tm1Dcl}$, $Egfr^{Wa5}/+$ pups were born at expected ratios and were normal in size at birth. However, these mice had reduced postnatal growth rates resulting in a noticeable decrease in body size by 1 week of age relative to their littermates. Similar to $Egfr^{wa2/Wa5}$ compound
30  mutants, $Tgfa^{tm1Dcl/tm1Dcl}$, $Egfr^{Wa5}/+$ double mutants showed an approximate 25% reduction in body weight by 6 weeks of age when compared with $Tgfa^{tm1Dcl}$ homozygous or $Egfr^{Wa5}$ heterozygous littermates. Additionally, two $Tgfa^{tm1Dcl/tm1Dcl}$, $Egfr^{Wa5}/+$ double mutants showed smaller lower jaws.

Interestingly, unlike body weight, the coat phenotype of the double-mutant pups was not exacerbated and remained indistinguishable from $Egfr^{Wa5}/+$ or $Tgfa^{tm1Dcl/tm1Dcl}$ single-mutant mice.

EgfrWa5 allele reduces the multiplicity but not growth of ApcMin-
5    meditated intestinal tumors. It has been previously showed that $Apc^{Min}$-mediated intestinal tumor multiplicity is dramatically reduced in $Egfr^{wa2/wa2}$ mice (Roberts et al., 2001). Therefore, to functionally verify the reduced activity of $Egfr^{Wa5}$, $Apc^{Min}/+$ mice were crossed with $Egfr^{Wa5}/+$ mice to generate $Apc^{Min}/+$ $Egfr^{Wa5}/+$ double heterozygotes. Three month-old
10    $Apc^{Min}/+$ $Egfr^{Wa5}/+$ mice had a 46% reduction in the average number of macroscopic intestinal polyps (> 0.3 mm) compared with $Apc^{Min}/+$ littermates wild type for Egfr ($Egfr^{Wa5}/+$; 7.4 ± 1.7; Egfr+/+; 13.7 ± 2.6; p = 0.03; see Figure 2A). Since the cross was segregating alleles at the major $Apc^{Min}$ modifier locus Mom1, each mouse was genotyped for resistance (r) or
15    susceptibility (s) at the Mom1 locus. On a $Mom1^{r/s}$ heterozygous background, $Apc^{Min}/+$ $Egfr^{Wa5}/+$ mice showed 53% fewer intestinal polyps than $Apc^{Min}/+$ mice, whereas on a $Mom1^{s/s}$ background, $Apc^{Min}/+$ $Egfr^{Wa5}/+$ mice showed a 32% reduction in tumor number when compared with $Apc^{Min}/+$. $Apc^{Min}/+$ mice on a $Mom1^{r/s}$ background showed a 42% reduction
20    in polyp multiplicity, independent of $Egfr^{Wa5}$ status, compared with a $Mom1^{s/s}$ background (p = 0.03), consistent with a previous report (Gould et al., 1996). No gender-dependent differences in polyp multiplicity were observed.

As disclosed previously for $Egfr^{wa2}$, mice carrying one $Egfr^{Wa5}$ allele caused no reduction in tumor size; the average polyp diameter in $Apc^{Min}/+$,
25    $Egfr^{Wa5}/+$ mice was 0.97 ± 0.07 mm compared with 1.05 ± 0.04 mm in $Apc^{Min}/+$ mice (p = 0.17; see Figure 2B). $Apc^{Min}/+$ mice on a $Mom1^{r/s}$ background showed a 16% reduction in polyp size, independent of $Egfr^{Wa5}$ status, compared with a $Mom1^{s/s}$ background (p = 0.0003) as previously reported (Gould et al.,
30    1996). There were no significant differences in polyp size related to gender.

In vivo tyrosine kinase activity is attenuated in $Egfr^{Wa5}$ heterozygous mutants. Exposure of exogenous EGF rapidly induces tyrosine phosphorylation of EGFR, along with 55- and 120-kD proteins, in multiple

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tissues of newborn mice (Donaldson & Cohen, 1992; Luetteke *et al.*, 1994). Although high doses of EGF induce equivalent EGFR phosphorylation in *Egfr*$^{wa2/wa2}$ and *Egfr* wild type neonates, low doses of exogenous EGF result in diminished EGFR phosphorylation in *Egfr*$^{wa2/wa2}$ mice, particularly in skin

5      (Luetteke *et al.*, 1994). In order to characterize the in vivo phosphorylation levels of the *Egfr*$^{Wa5}$ mutant receptor, wild type and *Egfr*$^{Wa5}$/+ littermates were injected subcutaneously with either PBS or EGF at 2 or 7 days of age. Similar to EGFR activity in *Egfr*$^{wa2/wa2}$ mice, phosphorylation levels of EGFR and other proteins in *Egfr*$^{Wa5}$/+ mice were greatly attenuated at lower doses

10     of EGF (0.5 or 1.0 µg/g body weight) in liver and skin (*see* Figure 3A). However, at pharmacological doses of EGF (10 µg/g body weight), tyrosine phosphorylation of EGFR and the 55-kD protein was also greatly attenuated in *Egfr*$^{Wa5}$/+ mice (*see* Figure 3B), in contrast to the normal level of tyrosine phosphorylation previously observed in *Egfr*$^{wa2/wa2}$ mice (Luetteke *et al.*,

15     1994). Total levels of EGFR were identical between wild type and *Egfr*$^{Wa5}$/+ mice, indicating that attenuation of tyrosine phosphorylation in *Egfr*$^{Wa5}$/+ is not due to differential receptor expression but to an inhibitory affect of the Wa5 receptor on wild type EGFR.

            *Egfr*$^{wa5}$ codes for a kinase-dead, dominant negative receptor. All *in*

20     *vivo* analyses suggested that the *Egfr*$^{wa5}$ allele reduces total EGFR activity, even when heterozygous with an *Egfr* wild type allele. In stark contrast, the *Egfr*$^{tm1Mag}$ null and *Egfr*$^{wa2}$ hypomorphic alleles have no detectable heterozygous phenotype. Thus, *Egfr*$^{Wa5}$ satisfies the genetic definition of an antimorphic allele and would be one of the few documented mammalian

25     antimorphic alleles; the other validated examples are the $T^{c}$, *Clock*$^{m1Jt}$, *Crebbp*$^{Gt(U-San)112Imeg}$, and *Fbn1*$^{Tsk}$ alleles (MacMurray & Shin, 1988; King *et al.*, 1997; Oike *et al.*, 1999; Gayraud *et al.*, 2000). Therefore, to assess the mechanism by which *Egfr*$^{Wa5}$ inhibits wild type EGFR signaling, wild type human *EGFR*, *EGFR*$^{kd}$, and *EGFR*$^{Wa5}$ expression vectors were transiently

30     transfected into CHO cells for *in vitro* analysis. The *EGFR*$^{kd}$ mutation was used as a control for an inactive kinase as this mutant cannot utilize ATP and thus is kinase dead (Honegger *et al.*, 1987). Since CHO cells lack endogenous detectable EGFR, total EGFR levels proportionately increased

in wild type, kd, and Wa5 EGFR-expressing cells with increasing quantities of their respective expression vector (Figure 4A), indicating that the mutations in $EGFR^{kd}$ and $EGFR^{Wa5}$ do not impair EGFR translation. Because CHO cells express endogenous EGFR ligands, the phosphorylation of wild type EGFR increased proportionately depending on the quantity of transfected expression vector in the absence of additional exogenous EGF treatment. In contrast, the kd and Wa5 mutants had no detectable phosphorylation, suggesting that they are inactive kinases.

Since receptor dimerization is essential for activation of EGFR kinase activity under normal conditions, cross-linking assays using wild type, kd and Wa5 EGFR expressing cells were performed to determine whether Wa5 has an inactive kinase due to an inability to form ligand-dependent dimers. CHO cells were transiently transfected with 0.5 $\mu$g of each expression vector and treated 24 hours later with the cross-linking agent BS3. EGFR and the two mutants forms, kd and Wa5, underwent equivalent levels of homodimerization indicating that neither mutation affected dimerization (Figure 4B). Dimers with the two mutants showed absence of tyrosine phosphorylation, suggesting that Wa5 might function as a dominant negative receptor.

The effect of co-expressing mutant receptors with wild type EGFR to determine the mechanism underlying the $Egfr^{Wa5}$ antimorphic allele was then analyzed. Wild type EGFR expression vector (0.2 $\mu$g) was co-transfected individually or with half (0.1 $\mu$g) or equal (0.2 $\mu$g) molar ratios of wild type $EGFR$, $EGFR^{kd}$, or $EGFR^{Wa5}$ expression vectors. The total amount of expression vector used was in the linear range of total EGFR expression (Figure 4A). Total EGFR levels increased proportionately with increasing quantities of co-transfected vector for each of the three expression vectors (Figure 4C). Similarly, EGFR phosphorylation levels increased proportionately with the quantity of wild type EGFR expression vector when transfected alone. However, phosphorylation of total EGFR remained constant in cells co-transfected with the $EGFR^{kd}$ expression vector despite increasing levels of total EGFR, suggesting that the kd mutant, although kinase dead, does not inhibit the activity of wild type $EGFR$ when co-

transfected with an equal molar ratio. Interestingly, phosphorylation of total EGFR decreased in proportion to the level of $Egfr^{Wa5}$ expression vector. Less than 10% of wild type EGFR phosphorylation levels was detected in the cells co-transfected with equal molar ratios of the $EGFR^{Wa5}$ and wild type

5    $EGFR$ expression vectors compared with wild type EGFR alone.  This result establishes that the Wa5 receptor can potently inhibit activation of wild type EGFR. Furthermore, this result suggests that EGFR inhibition by the Wa5 mutant is due to a novel mechanism since the kd mutant, which also lacks a functional kinase, did not inhibit EGFR activation; and since classical

10   cytoplasmic-tail truncated dominant negative receptors require over-expression to inhibit EGFR signaling (Xie *et al.*, 1997).

To directly compare the phosphorylation status of EGFR-EGFR, EGFR-kd and EGFR-Wa5 complexes, the FLAG (f) epitope was employed to tag the N-termini of the mature receptors, after the signal peptide. CHO cells

15   were transiently co-transfected with wild type $EGFR$ expression vector and an equal quantity of wild type $EGFR^{f}$, $EGFR^{fkd}$, or $EGFR^{fWa5}$ FLAG-tagged expression vectors. This permitted an analysis of the phosphorylation status of fEGFR, fkd, and fWa5 after immunoprecipitation of cell extracts using an anti-FLAG antibody (Figure 4D). The three transfections had similar levels of

20   EGFR and FLAG reactivity after immunoprecipitation with an anti-FLAG antibody indicating they had similar expression of FLAG-tagged protein in each transfection. However, the phosphorylation of fkd was dramatically reduced in cells co-transfected with $EGFR$ and $EGFR^{fkd}$ expression vectors suggesting that two active kinases are required for optimal EGFR activation

25   and that kd is phosphorylated, albeit at reduced levels, by EGFR. Nonetheless, dimerization of fkd and EGFR still resulted in phosphorylation indicating that EGFR-fkd homodimers have kinase activity.  Conversely, phosphorylation was not detected in EGFR-fWa5 complexes indicating that Wa5 cannot be phosphorylated by EGFR and that EGFR-Wa5 homodimers

30   lack kinase activity. EGFR expression vectors with C-terminal FLAG tags exhibit similar results to the N-terminal tagged proteins showing that the location of the FLAG tag does not adversely affect activation of EGFR.

The results using co-transfections with wild type EGFR suggested that Wa5 inhibits EGFR activation by inducing or preventing a conformational change that inactivates the EGFR kinase domain or by preventing the receptor from becoming a substrate in a transphosphorylation reaction. To distinguish between these two potential mechanisms, the hyper-activatable Dsk5 was employed, produced by the $EGFR^{Dsk5}$ hypermorph allele (Fitch *et al.*, 2003). Co-transfection of $EGFR^{Dsk5}$ and $EGFR^{Wa5}$ expression vectors revealed that Wa5 does not inhibit Dsk5 kinase activity (Figure 4E): Wa5 can be a substrate for Dsk5. EGFR phophorylation increased proportionally to the quantity of $EGFR^{Wa5}$ expression vector co-transfected with $EGFR^{Dsk5}$ suggesting that Wa5 was phosphorylated by hyper-activatable Dsk5. This result provides evidence that Wa5 inhibits wild type EGFR by disrupting kinase activation within oligomeric complexes rather than by inducing a conformational change leading to defective substrate utilization.

## Example 8
### $EGFR^{Wa5}$ does not Inhibit EGFR/ERBB2 Heterodimeric Complexes

Similar to Dsk5, the kinase of the EGFR-related protein, ERBB2, has much greater activity with constitutive activation when over-expressed. Since ERBB2 is the preferential heterodimerization partner for EGFR and other ERBB receptors (Graus-Porta *et al.*, 1997), whether Wa5 also inhibits heterodimeric complexes with ERBB2 was investigated. *ERBB2* expression vector (0.1 mg) was co-transfected individually or with 0.1 µg, 0.3 µg or 0.5 µg of *EGFR*, $EGFR^{kd}$ or $EGFR^{Wa5}$ expression vectors. Total EGFR levels were proportionally increased with increasing quantities of co-transfected vector for all three *EGFR* expression vectors (Figure 5A). However, ERBB2 levels decreased proportionally with increasing quantities of co-transfected *EGFR* expression vector. Co-transfection of $ERBB2^{K732M}$, coding for a kinase dead mutant of ERBB2, and $EGFR^{kd}$ expression vectors exhibited similar results showing that the reduction in ERBB2 levels was independent of the phosphorylation status of either EGFR or ERBB2, and most likely is a

result of transcriptional interference or altered intracellular trafficking as previously reported (Worthylake & Wiley, 1997).

Phosphorylation of both EGFR and ERBB2 increased in cells co-transfected with wild type *EGFR* and *ERBB2* expression vectors in proportion to the level of total EGFR (Figure 5A). However, with *EGFR^{kd}* and *EGFR^{Wa5}* co-transfections, the level of ERBB2 phosphorylation was dependent on the level of ERBB2 and was not reduced by increasing levels of kd or Wa5. Interestingly, co-transfection of *ERBB2* and either *EGFR^{kd}* or *EGFR^{Wa5}* expression vectors resulted in phosphorylation of kd and Wa5, respectively (Figure 5A), although kd and Wa5 alone did not exhibit phosphorylation (Figure 4A). The respective receptors were also immunoprecipitated for western blot analysis to confirm this result since separation of EGFR and ERBB2 by SDS-PAGE is often ambiguous in cells overexpressing both receptors. Equal quantities of *ERBB2* expression vector (0.1 µg) was co-transfected with 0.3 µg or 0.5 µg of wild type *EGFR*, *EGFR^{kd}*, or *EGFR^{Wa5}* expression vectors followed by immunoprecipitation of the cell extracts with either anti-EGFR or anti-ERBB2 antibodies (Figure 5B). The immunoprecipitation-western blot analysis produced a similar result as shown in Figure 5A (draft); Wa5 phosphorylation levels were slightly reduced compared to that of kd when controlled for EGFR levels. However, both EGFR mutants exhibited phosphorylation indicating that ERBB2 phosphorylates these EGFR mutants and that ERBB2 kinase activity is not inhibited by either kd or Wa5.

## Discussion of Example 8

Since the *in vivo* and *in vitro* data strongly suggests that a simple dimer model cannot explain the potent antimorphic activity of *Egfr^{Wa5}*, it is suggested that an alternative tetrameric or oligomeric model is more consistent with the activity of Wa5. Structural analysis of protein kinases in "on" and "off" states has revealed that conformational plasticity is central to the mechanism of kinase activity regulation. Autophosphorylation within the activation loop of the kinase domain causes conformational changes, removing substrate binding inhibition and properly positioning the catalytic

groups (Huse & Kuriyan, 2002). However, the tyrosine kinase domain of EGFR is highly unusual in that phosphorylation of the activation loop does not occur to promote its activity (Gotoh *et al.*, 1992). The crystal structure of the unphosphorylated EGFR kinase domain suggests that the EGFR

5   activation loop adopts a conformation observed only in other kinases that are phosphorylated and activated (Stamos *et al.*, 2002).

Although it is well established that ligand-induced dimerization increases the tyrosine kinase activity of EGFR, the mechanism by which EGFR kinase is activated is not clearly understood (Schlessinger, 2000). It

10  is thought that ligand-stabilized extracellular dimerization induces cytoplasmic dimerization in which the activation loops are stabilized in a conformation favorable for catalysis. The results disclosed herein suggest that EGFR-Wa5 dimers do not support a conformation necessary for catalysis and thus Wa5 acts as dominant negative like EGFR truncation

15  mutants lacking most of the cytoplasmic domain (Kashles *et al.*, 1991). A simple dimmer model would suggest that $EGFR^{Wa5}/+$ cells should have at least 25% of wild type activity, much greater than what was actually observed. Rather, similar to the dominant-negative effect of the $KIT^{E839K}$ mutant on wild type KIT phosphorylation (Longley *et al.*, 1999), a more

20  drastic reduction of phosphorylation in CHO cells suggests that EGFR-Wa5 dimers may interfere with kinase activity of wild type dimers through tetramer or high-order inter-molecular process between ligand-stabilized dimers (Sherrill, 1997; *see* Figure 6A).

Consequently, a model that is more consistent with the present

25  analysis of Wa5 consists of membrane-bound pre-dimers, stabilized by ligand binding, that are not yet fully activated. Rather than the 25% of normal kinase activity level predicted in $EGFR^{Wa5}/+$ cells based upon a dimer model, substantially less wa observed, lower than 10% activity, suggesting that the signal has been further diluted. More consistent with this

30  observation is tertameric or high-order complexes underlying the activated receptors (Honegger *et al.*, 1989; Murali *et al.*, 1996; Huang *et al.*, 1998). Using a tetramer model, only one-sixteenth of the complexes would be fully active; all others would contain at least one Wa5 receptor, thus disrupting full

activation of the receptor complexes. Therefore, the currently proposed model suggests that dimers become stabilized upon ligand binding, but must aggregate to become fully activated. Upon activation, they could then efficiently propagate a signaling cascade.

5          Consistent with this model would be a quarternary complex that is required to activate members within the respective dimers of the tetrameric complex and that these complexes are essentially kinase dead when containing a Wa5 receptor and incapable of further aggregation to activate EGFR-Wa5 dimers. For example, upon stabilization of dimers by ligand

10    binding, the dimers might be internalized and it is within cytoplasmic vesicles that these higher order complexes might form (Wang et al., 2002; Wiley, 2003). Removal from the cell surface provides one potential mechani sm by which Wa5 can permanently inhibit complexes containing wild type receptors and preventing disassociation and reassociation of wild type EGFR d imers.

15    Although it cannot be formally ruled out that Wa5 forms stable, inactive dimers with EGFR (Figure 6A), this instantly disclosed data suggests otherwise. A greater level of interaction between EGFR and Wa5 in the FLAG-tagged experiments would have been expected to have been observed if this were the case.

20          The model proposed herein for homodimeric interactions is also consistent with heterodimeric interactions (Figure 6B). Previous studies have suggested that ERBB2 remains in cytoplasmic vesicles in non-transformed cells while EGFR is predominately on the cell surface (Worthylake & Wiley, 1997), consistent with the lack a known ligand for

25    ERBB2. Upon EGFR activation, ERBB2 becomes trans-phosphorylated, most likely by internalized vesicles containing ligand-stabilized EGFR dimers. This model also suggests that EGFR and ERBB2 do not form stabilized dimers but rather are part of tetrameric or higher-order complexes. Furthermore, this model is consistent with lateral signal propagation between

30    activated receptors (Graus-Porta et al., 1997). Lastly, phosphoryla tion of Wa5 by ERBB2 suggests that Wa5 does not inhibit these highe r-order heteromeric complexes when highly active kinases are present. Thus, this is

also consistent with the inability of Wa5 to inhibit Dsk5 mutant receptors that act very similar to ERBB2.

## Example 9

### Preparation of a Recombinant Peptide Library in Phage

A population of DNA fragments encoding recombinant peptide sequences is cloned into the T7 SELECT™ vector (Novagen, Madison, Wisconsin, United States of America). Cloning at the Eco RI restriction enzyme recognition site places the recombinant peptide in-frame with the 10B protein such that the peptide is displayed on the capsid protein. The resulting reading frame requires an AAT initial codon followed by a TCX codon.

The molar ratio between insert and vector is 1:1. Size-fractionated cDNA inserts are prepared by gel filtration on sepharose 4B and ranged from 27 base pairs to 33 base pairs. cDNAs are ligated by use of the DNA ligation kit (Novagen, Madison, Wisconsin, United States of America). Recombinant T7 DNA is packaged according to the manufacturer's instructions and amplified prior to bioscreening in animal tumor models. The diversity of the library can be at least $10^6$.

## Example 10

### Production of a Phage-Displayed scFv Antibody Library

A phage-displayed antibody library is constructed based upon previously published methodologies (see Pope et al., 1996). Briefly, spleens from outbred newborn and three-to-four week old mice and rats are used as a source of antibody-encoding genetic material to produce a library of about $2 \times 10^9$ members. The antibody-encoding genetic material is cloned into the pCANTAB phagemid vector.

The pCANTAB vector contains an amber stop codon that is located downstream of the scFv coding sequences and upstream of the M13 gene III coding sequences. E. coli TG1 cells (a supE strain of E. coli) contain a suppressor tRNA that inserts a glutamic acid residue in response to an UAG (amber) stop codon. The amber stop codon is about 14% efficient.

Therefore, the scFv antibody amino acid sequences will be fused to M13 phage gene III amino acid sequences about 14% of the time, and will be produced as a soluble, non-fusion protein about 86% of the time when the library is grown in TG1 cells. In contrast, *E. coli* strain HB2151 does not

5   contain the amber stop codon, and thus only soluble non-fused scFv will be produced when the library is grown in HB2151.


## References

The references listed below as well as all references cited in the
10   specification are incorporated herein by reference to the extent that they supplement, explain, provide a background for or teach methodology, techniques and/or compositions employed herein.

Adams GP (1998) Improving the tumor specificity and retention of antibody-based molecules. *In Vivo* 12:11-21.

15   Adams GP, McCartney JE, Wolf EJ, Eisenberg J, Tai MS, Huston JS, Stafford WF 3rd, Bookman MA, Houston LL & Weiner LM (1995) Optimization of *in vivo* tumor targeting in SCID mice with divalent forms of 741F8 anti-c-ERBB2 single-chain Fv: effects of dose escalation and repeated i.v. administration. *Cancer Immunol*

20   *Immunother* 40:299-306.

Altschul SF, Gish W, Miller W, Myers EW & Lipman DJ (1990) Basic Local Alignment Search Tool. *J Mol Biol* 215:403-410.

Andersson L, Blomberg L, Flegel M, Lepsa L, Nilsson B & Verlander M (2000) Large-scale synthesis of peptides. Biopolymers 55:227-250.

25   Apostolakis EM, Garai J, Lohmann JE, Clark JH &O'Malley BW (2000) Epidermal growth factor activates reproductive behavior independent of ovarian steroids in female rodents. *Mol Endocrinol* 14:1086-1098.

Ausubel F, ed (1995) Short Protocols in Molecular Biology, 3rd ed. Wiley, New York, United States of America.

30   Barton GJ (1998) Protein Sequence Alignment Techniques. *Acta Crystallogr D Biol Crystallogr* 54:1139-1146.

Batzer MA, Carlton JE & Deininger PL (1991) Enhanced evolutionary PCR using oligonucleotides with inosine at the 3'- terminus. *Nucleic Acids Res* 19:5081.

Better M, Chang CP, Robinson RR & Horwitz AH (1988) *Escherichia coli* secretion of an active chimeric antibody fragment. *Science* 240:1041-3.

Bodanszky M (1993) Principles of Peptide Synthesis, 2nd rev. ed. Springer-Verlag, New York, New York, United States of America.

Cai X & Garen A (1995) Anti-melanoma antibodies from melanoma patients immunized with genetically modified autologous tumor cells: selection of specific antibodies from single-chain Fv fusion phage libraries. *Proc Natl Acad Sci U S A* 92:6537-41.

Carpenter G & Wahl MI (1990) The epidermal growth factor family. *In* Handbook of Experimental Pharmacology, Peptide Growth Factors and Their Receptors I (ed. Sporn MB & Roberts AB), pp. 69-171. Springer-Verlag, New York, New York, United States of America.

Cipolla DC, Gonda I, Shak S, Kovesdi I, Crystal R & Sweeney TD (2000) Coarse Spray Delivery to a Localized Region of the Pulmonary Airways for Gene Therapy. *Hum Gene Ther* 11:361-371.

Coker KJ, Staros JV & Guyer CA (1994) A kinse-negative epidermal growth factor receptor that retains the capacity to stimulate DNA synthesis. *Proc Natl Acad Aci U S A* 91:6967-6971.

Corringer PJ, Weng JH, Ducos B, Durieux C, Boudeau P, Bohme A & Roques BP (1993) CCK-B agonist or antagonist activities of structurally hindered and peptidase-resistant Boc-CCK4 derivatives. *J Med Chem* 36:166-172.

Coussens L, Yang-Feng TL, Liao Y-C, Chen E, Gray A, *et al.,* (1985) Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. *Science* 230:1132-1139.

Donaldson, RW & Cohen S (1992) Epidermal growth factor stimulates tyrosine phosphorylation in the neonatal mouse: association of a M(r)

55,000 substrate with the receptor. *Proc Natl Acad Sci U S A* 89:8477-81.

Egger B, Buchler MW, Lakshmanan J, Moore P & Eysselein VE (2000) Mice harboring a defective epidermal growth factor receptor (waved-2) have an increased susceptibility to acute dextran sulfate-induced colitis. *Scand J Gastroenterol* 35:1181-7.

Fields GB & Noble RL (1990) Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. *Int J Pept Protein Res* 35:161-214.

Figini M, Obici L, Mezzanzanica D, Griffiths A, Colnaghi MI, Winter G & Canevari S (1998) Panning phage antibody libraries on cells: isolation of human Fab fragments against ovarian carcinoma using guided selection. *Cancer Res* 58:991-6.

Fitch, KR, McGowan KA, van Raamsdonk CD, Fuchs H, Lee D, Puech A, Herault Y, Threadgill DW, Hrabe de Angelis M & Barsh GS (2003) Genetics of dark skin in mice. *Genes Dev* 17:214-28.

Garbay-Jaureguiberry C, Ficheux D & Roques BP (1992) Solid phase synthesis of peptides containing the non-hydrolysable analog of (O)phosphotyrosine, p(CH2PO3H2)Phe. Application to the synthesis of 344-357 sequences of the beta 2 adrenergic receptor. *Int J Pept Protein Res* 39:523-527.

Gayraud B, Keene DR, Sakai LY & Ramirez F (2000) New insights into the assembly of extracellular microfibrils from the analysis of the fibrillin 1 mutation in the Tight skin mouse. *J Cell Biol* 150:667–680.

Glover DM & Hames BD (1995) <u>DNA Cloning : A Practical Approach</u>, 2nd ed. IRL Press at Oxford University Press, Oxford, England.

Gotoh, N, Tojo A, Hino M, Yazaki Y & Shibuya M (1992) A highly conserved tyrosine residue at codon 845 within the kinase domain is not required for the transforming activity of human epidermal growth factor receptor. *Biochem Biophys Res Commun* 186:768-74.

Gould, KA, Dietrich WF, Borenstein N, Lander ES & Dove WF (1996) Mom1 is a semi-dominant modifier of intestinal adenoma size and multiplicity in Min/+ mice. *Genetics* 144:1769-1776.

Graus-Porta, D, Beerli RR, Daly JM & Hynes NE (1997) ERBB2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. *EMBO J* 16:1647-1655.

Gullick WJ (1998) Type I growth factor receptors: current status and future work. *Biochem Soc Symp* 63:193-198.

Habib NA, Hodgson HJ, Lemoine N & Pignatelli M (1999) A Phase I/Ii Study of Hepatic Artery Infusion with wtp53-CMV-Ad in Metastatic Malignant Liver Tumours. *Hum Gene Ther* 10:2019-2034.

Hanks SK, Quinn AM & Hunter T (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* 241:42-52.

Helmrath MA, Erwin CR & Warner MD (1997) A defective EGF-receptor in waved-2 mice attenuates intestinal adaptation. *J Surg Res* 69:76-80.

Henikoff JG, Pietrokovski S, McCallum CM & Henikoff S (2000) Blocks-Based Methods for Detecting Protein Homology. *Electrophoresis* 21:1700-1706.

Henikoff S & Henikoff JG (2000) Amino Acid Substitution Matrices. *Adv Protein Chem* 54:73-97.

Henikoff S & Henikoff JG (1992) Amino Acid Substitution Matrices from Protein Blocks. *Proc Natl Acad Sci U S A* 89:10915-10919.

Hoogenboom HR & Winter G (1992) By-passing immunisation. Human antibodies from synthetic repertoires of germline VH gene segments rearranged *in vitro*. *J Mol Biol* 227:381-8.

Honegger AM, Dull TJ, Felder S, Van Obberghen E, Bellot F, Szapary D, Schmidt A, Ullrich A & Schlessinger J (1987) Point mutation at the ATP binding site of EGF receptor abolishes protein-tyrosine kinase activity and alters cellular routing. *Cell* 51: 199-209.

Honegger AM, Kris RM, Ullrich A & Schlessinger J (1989) Evidence that autophosphorylation of solubilized receptors for epidermal growth factor is mediated by intermolecular cross-phosphorylation. *Proc Natl Acad Aci U S A* 86:925-929.

Huang CC, Novak WR, Babbitt PC, Jewett AI, Ferrin TE & Klein TE (2000) Integrated Tools for Structural and Sequence Alignment and Analysis. *Pac Symp Biocomput*:230-241.

Huang GC, Ouyang X & Epstein RJ (1998) Proxy activation of protein ERBB2 by heterologous ligands imllies a heterotetrameric mode of receptor tyrosine kinase interaction. *Biochem J* 331:113-119.

Huse M & Kuriyan J (2002) The conformational plasticity of protein kinases. *Cell* 109:275-282.

Jones PT, Dear PH, Foote J, Neuberger MS & Winter G (1986) Replacing the complementarity-determining regions in a human antibody with those from a mouse. *Nature* 321:522-525.

Karlin S & Altschul SF (1993) Applications and Statistics for Multiple High-Scoring Segments in Molecular Sequences. *Proc Natl Acad Sci U S A* 90:5873-5877.

Kashles O, Yarden J, Fischer R, Ullrich A & Schlessinger J (1991) A dominant negative mutation suppresses the function of normal epidermal growth factor receptors by heterodimerization. *Mol Cell Biol* 11:1454-1463.

Kim J, Ahn S, Guo R & Daaka Y (2003) Regulation of epidermal growth factor receptor internalization by G protein-coupled receptors. *Biochem* 42:2887-94.

King DP, Vitaterna MH, Chang AM, Dove WF, Pinto LH, Turek FW & Takahashi JS (1997) The mouse Clock mutation behaves as an antimorph and maps within the W19H deletion, distal of Kit. *Genetics* 146:1049-1060.

Kraus MH, Issing W, Miki T, Popescu NC & Aaronson SA (1989) Isolation and characterization of ERBB3, a third member of the ERBB/epidermal growth factor receptor family: evidence for overexpression in a subset of human mammary tumors. *Proc Natl Acad Sci U S A* 86:9193-9197.

Kroiher, M, Miller MA & Steele RE (2001) Deceiving appearances: signaling by "dead" and "fractured" receptor protein-tyrosine kinases. *Bioessays* 23:69-76.

Kyte J & Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. *J Mol Biol* 157:105-132.

Law B (1996) Immunoassay: A Practical Guide. Taylor & Francis, London, England.

5    Lee D, Cross SH, Strunk KE, Morgan JE, Bailey CL, Jackson IJ & Threadgill DW (2004) *Wa5* is a novel ENU-induced antimorphic allele of the epidermal growth factor receptor. *Mamm Genome* 15:525-36.

Longley BJ, Jr, Metcalfe DD, Tharp M, Wang X, Tyrrell L, Lu SZ, Heitjan D & Ma Y (1999) Activating and dominant inactivating c-KIT catalytic

10       domain mutations in distinct clinical forms of human mastocytosis. *Proc Natl Acad Sci U S A* 96:1609-14.

Lu Z, Murray KS, Van Cleave V, LaVallie ER, Stahl ML & McCoy JM (1995) Expression of thioredoxin random peptide libraries on the Escherichia coli cell surface as functional fusions to flagellin: a system designed

15       for exploring protein-protein interactions. *Biotechnology* (NY) 13:366-372.

Luetteke NC, Phillips HK, Qiu TH, Copeland NG, Earp HS, Jenkins NA & Lee DC (1994) The mouse waved-2 phenotype results from a point mutation in the EGF receptor tyrosine kinase. *Genes Dev* 8:399-413.

20    Luetteke NC, Qiu TH, Peiffer RL, Oliver P, Smithies O & Lee DC (1993) TGF alpha deficiency results in hair follicle and eye abnormalities in targeted and waved-1 mice. *Cell* 73:263-278.

MacMurray A & Shin HS (1988) The antimorphic nature of the Tc allele at the mouse T locus. *Genetics* 120:545-550.

25    Mann GB, Fowler KJ, Gabriel A, Nice EC, Williams RL & Dunn AR (1993) Mice with a null mutation of the TGF alpha gene have abnormal skin architecture, wavy hair, and curly whiskers and often develop corneal inflammation. *Cell* 73:249-61.

Manson MM (1992) Immunochemical Protocols. Humana Press, Totowa,

30       New Jersey, United States of America.

McCafferty J, Griffiths AD, Winter G & Chiswell DJ (1990) Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* 348:552-4.

McOmie JFW.    (1973) Protective Groups in Organic Chemistry, Plenum Press, London, England.

Merrifield RB (1969) Solid-phase peptide synthesis.  *Adv Enzymol Relat Areas Mol Biol* 32:221-296.

Miettinen PJ, Chin JR, Shum L, Slavkin HC, Shuler CF, Derynck R & Werb Z (1999) Epidermal growth factor receptor function is necessary for normal craniofacial development and palate closure. *Nature Genetics* 22:69-73.

Mocikat R, Kutemeier G, Hoffmann-Fezer G & Thierfelder S (1994) A mouse model for the preclinical evaluation of immunosuppressive effector functions of human isotypes. The human IgG1 isotype is superior to IgG3. *Transplantation* 57:405-11.

Moscatello DK, Holgado-Madruga M, Godwin AK, Ramirez G, Gunn G, Zoltick PW, Biegel JA, Hayes RL & Wong AJ (1995) Frequent expression of a mutant epidermal growth factor receptor in multiple human tumors. *Cancer Res* 55:5536-9.

Moser AR, Mattes EM, Dove WF, Lindstrom MJ, Haag JD & Gould MN. (1993) ApcMin, a mutation in the murine Apc gene, predisposes to mammary carcinomas and focal alveolar hyperplasias. *Proc Natl Acad Sci U S A* 90:8977-81.

Murali R, Brennan PJ, Kieber-Emmons T & Greene MI (1996) Structural analysis of p185c-neu and epidermal growth factor receptor tyrosine kinases: oligomerization of kinase domains. *Proc Natl Acad Sci U S A* 93:6252-7.

Needleman SB & Wunsch CD (1970) A General Method Applicable to the Search for Similarities in the Amino Acid Sequence of Two Proteins. *J Mol Biol* 48:443-453.

Ohtsuka E, Matsuki S, Ikehara M, Takahashi Y & Matsubara K (1985) An alternative approach to deoxyoligonucleotides as hybridization probes by insertion of deoxyinosine at ambiguous codon positions.  *J Biol Chem* 260:2605-2608.

Oike Y, Hata A, Mamiya T, Kaname T, Noda Y, Suzuki M, Yasue H, Nabeshima T, Araki K & Yamamura K (1999) Truncated CBP protein

leads to classical Rubinstein-Taybi syndrome phenotypes in mice: implications for a dominant-negative mechanism. *Hum Mol Genet* 8:387–396.

Pavone V, Di Blasio B, Lombardi A, Maglio O, Isernia C, Pedone C, Benedetti E, Altmann E & Mutter M (1993) Non coded C alpha, alpha-disubstituted amino acids. X-ray diffraction analysis of a dipeptide containing (S)-alpha-methylserine. *Int J Pept Protein Res* 41:15-20.

Pearson WR & Lipman DJ (1988) Improved Tools for Biological Sequence Comparison. *Proc Natl Acad Sci U S A* 85:2444-2448.

Plowman GD, Culouscou J-M, Whitney GS, Green JM, Carlton GW, Foy L, Neubauer MG & Shoyab M (1993) Ligand-specific activation of HER4/p180erbB4, a fourth member of the epidermal growth factor receptor family. *Proc Natl Acad Sci U S A* 90:1746-1750.

Plowman GD, Whitney GS, Neubauer MG, Green JM, McDonald VL, Todaro GJ & Shoyab M (1990) Molecular cloning and expression of an additional epidermal growth factor receptor-related gene. *Proc Natl Acad Sci U S A* 87: 4905-4909.

Pluckthun A (1994) in The Pharmacology of Monoclonal Antibodies, vol. 113, pp. 269-315, Rosenburg & Moore (eds.), Springer-Verlag, New York, New York, United States of America.

Pope A, Pritchard K, Williams A, Roberts A, Hackett JR, Mandecki W & Johnson KS (1996) In vitro selection of a high affinity antibody to oestradiol using a phage display human antibody library. *Immunotechnology* 2:209-217.

Presta LG (1992) Antibody engineering. *Curr Op Struct Biol* 2:593-596.

Qian X, LeVea CM, Freeman JK, Dougall WC, & Greene MI (1994) Heterodimerization of epidermal growth factor receptor and wild-type or kinase deficient Neu: a mechanism of interreceptor kinase activation and transphosphorylation. *Proc Natl Acad Sci U S A* 91:1500-1504.

Rasheed BK, Wiltshire RN, Bigner SH & Bigner DD (1999) Molecular pathogenesis of malignant gliomas. *Curr Opin Oncol* 11:162-7.

Reiter JL, Threadgill DW, Eley GD, Strunk KE, Danielsen AJ, Schehl-Sinclair C, Pearsall RS, Green PJ, Yee D, Lampland AL, Balasubramaniam, Crossley TO, Magnuson TR, James CD & Maihle NJ (2001) Comparative genomic sequence analysis and isolation of human and mouse alternative EGFR transcripts encoding truncated receptor isoforms. *Genomics* 71:1-20.

Riechmann L, Clark M, Waldmann H & Winter G (1988) Reshaping human antibodies for therapy. *Nature* 332:323-329.

Roberts, RB, Min L, Washington MK, Olsen SJ, Settle SH, Coffey RJ & Threadgill DW (2001) Importance of epidermal growth factor receptor signaling in establishment of adenomas and maintenance of carcinomas during intestinal tumorigenesis. *Proc Natl Acad Sci U S A* 99:1521-1526.

Rossolini GM, Cresti S, Ingianni A, Cattani P, Riccio ML & Satta G (1994) Use of deoxyinosine-containing primers vs degenerate primers for polymerase chain reaction based on ambiguous sequence information. *Mol Cell Probes* 8:91-98.

Sambrook J & Russell D (2001) Molecular Cloning: A Laboratory Manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, United States of America.

Saqi MA, Wild DL & Hartshorn MJ (1999) Protein Analyst--a Distributed Object Environment for Protein Sequence and Structure Analysis. *Bioinformatics* 15:521-522.

Schlessinger J (2000) Cell signaling by receptor tyrosine kinases. *Cell* 103:211-25.

Schneider CH & Eberle AN (1993) Peptides, 1992 : Proceedings of the Twenty-Second European Peptide Symposium, September 13-19, 1992, Interlaken, Switzerland. Escom, Leiden, The Netherlands.

Schröder E & Lübke K (1965) The Peptides. Academic Press, New York, New York, United States of America.

Shalaby MR, Shepard HM, Presta L, Rodrigues ML, Beverley PC, Feldmann M & Carter P (1992) Development of humanized bispecific antibodies

reactive with cytotoxic lymphocytes and tumor cells overexpressing the HER2 protooncogene. *J Exp Med* 175:217-25.

Sherrill JM (1997) Self-phosphorylation of epidermal growth factor receptor is an intermolecular reaction. *Biochem* 36:12890-6.

5   Silhavy TJ, Berman ML, Enquist LW & Cold Spring Harbor Laboratory. (1984) Experiments with Gene Fusions.   Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, United States of America.

Smith GP (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science 228:1315-

10      1317.

Smith TF & Waterman M (1981) Comparison of Biosequences. *Adv Appl Math* 2:482-489.

Stamos J Sliwkowski MX & Eigenbrot C (2002) Structure of the epidermal growth factor receptor kinase domain alone and in complex with a 4-

15      anilinoquinazoline inhibitor. *J Biol Chem* 277:46265-46272.

Stewart JM & Young JD (1969) Solid Phase Peptide Synthesis, Freeman, San Francisco, California, United States of America.

Strunk KE, Amann V & Threadgill DW (2004) Phenotypic variation resulting from a deficiency of epidermal growth factor receptor in mice is

20      caused by extensive genetic heterogeneity that can be genetically and molecularly partitioned. *Genetics* 167:1821-32.

Thaung C, West K, Clark BJ, McKie L, Morgan JE, Arnold K, Nolan PM, Peters J, Hunter AJ, Brown SD, Jackson IJ & Cross SH (2002) Novel ENU-induced eye mutations in the mouse: models for human eye

25      disease. *Hum Mol Genet* 11:755-67.

Threadgill DW, Dlugosz AA, Hansen LA, Tennenbaum T, Lichti U, Yee D, LaMantia C, Mourton T, Herrup K, Harris RC, Barnard JA, Yuspa SH, Coffey RJ & Magnuson T (1995) Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. *Science*

30      269:230-234.

Tijssen P (1993) Laboratory Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Acid Probes.   Elsevier, New York, New York, United States of America.

- 72 -

Tung CH, Zhu T, Lackland H & Stein S (1992) An acridine amino acid derivative for use in Fmoc peptide synthesis. *Pept Res* 5:115-118.

Urge L, Otvos L, Jr., Lang E, Wroblewski K, Laczko I & Hollosi M (1992)
5      Fmoc-protected, glycosylated asparagines potentially useful as reagents in the solid-phase synthesis of N-glycopeptides. *Carbohydr Res* 235:83-93.

U.S. Patent No. 4,244,946

U.S. Patent No. 4,554,101

U.S. Patent No. 5,223,409

10    U.S. Patent No. 5,264,563

U.S. Patent No. 5,498,538

U.S. Patent No. 5,578,629

U.S. Patent No. 5,580,717

U.S. Patent No. 5,650,489

15    U.S. Patent No. 5,667,988

U.S. Patent No. 5,702,892

U.S. Patent No. 5,738,996

U.S. Patent No. 5,747,334

U.S. Patent No. 5,756,291

20    U.S. Patent No. 5,780,225

U.S. Patent No. 5,811,392

U.S. Patent No. 5,811,512

U.S. Patent No. 5,811,515

U.S. Patent No. 5,817,757

25    U.S. Patent No. 5,817,879

U.S. Patent No. 5,824,483

U.S. Patent No. 5,840,479

U.S. Patent No. 5,858,670

U.S. Patent No. 5,858,784

30    U.S. Patent No. 5,922,545

U.S. Patent No. 5,939,598

U.S. Patent No. 5,948,635

U.S. Patent No. 6,013,638

U.S. Patent No. 6,015,561

U.S. Patent No. 6,015,881

U.S. Patent No. 6,022,737

U.S. Patent No. 6,031,071

5   U.S. Patent No. 6,057,098

U.S. Patent No. 6,107,059

U.S. Patent No. 6,136,295

U.S. Patent No. 6,156,511

U.S. Patent No. 6,168,912

10  U.S. Patent No. 6,174,708

U.S. Patent No. 6,180,348

U.S. Patent No. 6,214,553

U.S. Patent No. 6,225,447

U.S. Patent No. 6,593,081

15  Wang ZQ, Chen JK, Wang SW, Moeckel G & Harris RC (2003) Importance of functional EGF receptors in recovery from acute nephrotoxic injury. *J Am Soc Nephrol* 14:3147-3154.

Wang Y, Pennock S, Chen X & Wang Z (2002) Endosomal signaling of epidermal growth factor receptor stimulates signal transduction

20  pathways leading to cell survival. *Mol Cell Biol* 22:7279-7290.

Wiley HS (2003) Trafficking of the ErbB receptors and its influence on signaling. *Exp Cell Res* 284:78-88.

Wong AJ, Ruppert JM, Bigner SH, Grzeschik CH, Humphrey PA, Bigner DS & Vogelstein B (1992)   Structural alterations of the epidermal

25  growth factor receptor gene in human gliomas. *Proc Natl Acad Sci U S A* 89:2965-9.

Worthylake R & Wiley HS (1997) Structural aspects of the epidermal growth factor receptor required for transmodulation of ERBB2/neu. *J Biol Chem* 272:8594-601.

30  Xie W, Paterson AJ, Chin E, Nabell LM & Kudlow JE (1997) Targeted expression of a dominant negative epidermal growth factor receptor in the mammary gland of transgenic mice inhibits pubertal mammary duct development. *Mol Endocrinol* 11:1766-81.

Yokota T, Milenic D, Whitlow M & Schlom J (1992) Rapid Tumor Penetration of a Single-Chain Fv and Comparison with Other Immunoglobulin Forms. *Cancer Res* 52:3402-3408.

5      It will be understood that various details of the presently disclosed subject matter can be changed without departing from the scope of the presently disclosed subject matter. Furthermore, the foregoing description is for the purpose of illustration only, and not for the purpose of limitation.

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